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THE UNIVERSITY OF ALBERTA

THE REGULATION OF SKELETAL MUSCLE MEMBRANE EXCITABILITY BY ACETYLCHOLINE.

BY

© LINDA L. BAMBRICK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF Ph.D.

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1986.

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ABSTRACT

In this study the role of acetylcholine (ACh) activity in the neuronal regulation of muscle ACh receptors and sodium (Na) channels was examined. Neuronal input to the muscle was interrupted by denervation, blockade of ACh release (with botulinum toxin; BoTX) or by spinal isolation of the motoneurons innervating the muscles. Numbers of ACh receptors and Na channels were quantified by the binding of the radioligands ^{125}I-alpha-bungarotoxin (^{125}I-BTX) and ^{3}H-saxitoxin (^{3}H-STX), respectively.

In adult rat muscle, denervation and BoTX blockade were equally effective in inducing muscle atrophy and increasing ^{125}I-BTX binding site numbers above their control levels. Numbers of ^{3}H-STX binding sites normalized by muscle weight or muscle protein were unchanged following either treatment. However, due to muscle atrophy, this finding is consistent with an actual decline in ^{3}H-STX binding site density. Therefore, the expression of both ACh receptors and Na channels in adult muscle is controlled by the nerve and this regulation is mediated by neuromuscular activity.

Spinal isolation of the motor unit abolishes the evoked ACh release while non-evoked release of ACh continues. It contrasts with BoTX blockade where a portion of the non-evoked release of ACh is also inhibited. In cats, the effects of spinal isolation were not always equivalent to denervation in elevating ACh receptor numbers after 3

weeks. After 8 months, ^{125}I-BTX binding was still high in the denervated muscles, but had recovered back down towards control levels in the spinal isolation muscles. The first finding, of the non-equivalence between the two treatments, suggested that the non-evoked release of ACh may be an important component of the trophic neuromuscular activity. Since the depolarization of the muscle membrane induced by ACh would increase as the muscle fibre diameter decreased with atrophy of the muscle, the second finding - that spinal isolation muscles recovered towards normal, low levels of ACh receptors with no concomitant recovery of muscle activity - could be explained by the hypothesis that the neuronal regulation of muscle ACh receptors is mediated via the muscle membrane depolarization consequent on ACh release

In developing rats, BoTX-treatment was always less effective than denervation in increasing ^{125}I-BTX or decreasing ^{3}H-STX binding site numbers. Due to their higher input resistances, longer ACh receptor open times and lower levels of ACh esterase, neonatal rat muscles would develop larger depolarizations of the membrane in response to spontaneous ACh release than would adult muscles. Therefore, it was suggested that the non-evoked ACh release not blocked by BoTX could be exerting a larger trophic influence in immature than in mature muscle.

It was concluded that the observed data could be explained by postulating a neuronal regulation of muscle

ACh receptors and Na channels mediated by ACh release and that different conditions, including muscle fibre size and maturity, would determine the relative importance of evoked and non-evoked release of ACh in this regulation.

Keywords: Acetylcholine receptor; denervation; development; botulinum toxin; neuromuscular activity; sodium channel.

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List of Abreviations

Acetylcholine:	ACh
Acetylcholinesterase:	AChE
Alpha-bungarotoxin:	BTX
Botulinum toxin:	BoTX
Calcium ion:	Ca
Dissociation constant:	Kd
d-tubocurarine:	dTC
Electromyogram:	EMG
Extensor digitorum longus muscle:	EDL
Intragastral:	ig
Intramuscular:	im
Intravenous:	iv
Maximum specific binding:	Bmax
Miniature endplate potential:	mepp
Saxitoxin:	STX
Scorpion toxin:	ScTX
Sodium ion:	Na
Subcutaneous:	sc
Tetrodotoxin:	TTX

Triceps surae muscle group without soleus, t.s.
(lateral and medial gastronemius and plantaris)

CHAPTER ONE: INTRODUCTION

1.1. GENERAL INTRODUCTION

Nicotinic acetylcholine (ACh) receptors and voltage-activated sodium (Na) channels in the sarcolemma of skeletal muscles allow the muscle to respond to ACh released by the motoneuron with a local depolarization followed by the rapid spread of a wave of excitation all along the muscle membrane. This initiates a concerted activation of the muscle contractile proteins. These two integral membrane proteins, the ACh receptor and the Na channel, are functional specializations of the sarcolemma enabling the muscle to respond to neural signals. The number, distribution and biochemical properties of the ACh receptor and the Na channel are influenced by the motoneuron. The primary objective of the current study was to describe the influence of innervation on the development and maintenance of ACh receptor and Na channel numbers and to evaluate the role of neuromuscular activity in this regulation.

In the mature muscle membrane there are high concentrations of ACh receptors opposite the nerve terminals at the neuromuscular junction. ACh receptor density in the extrajunctional membrane is very low (Diamond & Miledi, 1962; Fambrough & Rash, 1971; Fambrough & Hartzell, 1972; Bevan & Steinbach, 1977). These receptors are chemically-activated ionophores which respond to ACh with a postsynaptic depolarization

consequent on the movement of ions through the open ACh receptor ion channel. In mature skeletal muscle, this depolarization activates Na channels and the excitation is propagated along the muscle membrane. The depolarization mobilizes calcium (Ca) which, in turn, interacts with muscle contractile proteins and induces muscle fibre contraction. The density of Na channels is highest near the neuromuscular junction (Beam, Caldwell & Campbell, 1985) although appreciable numbers of channels are found along the fibre surface membrane and in the t-tubular membrane (Jaimovich, Venosa, Shrager & Horowicz, 1976; Jaimovich, Chicheportiche, Lombet, Lazdunski, Ildefonse & Rougier, 1983; Jaimovich, Donosa, Liberona & Hildago, 1986).

Following denervation of the muscle, ACh receptors appear in the extrajunctional muscle membrane (Reid & Williams, 1949; Axelsson & Thesleff, 1959; Miledi & Potter, 1971; Hartzell & Fambrough, 1972; Colquhoun, Rang & Ritchie, 1974; Brockes & Hall, 1975; Askmark, Aquilonius, Eckernas, Fawcett & Nordberg, 1982; Askmark, Aquilonius, Fawcett, Nordberg & Eckernas, 1982; Mong, Farach & Martinez-Carrion, 1982; Pumplin & Fambrough, 1982; Kallo and Steinhardt, 1983; Goldman, Boulter, Heinemann and Patrick, 1985). These new ACh receptors have mean channel open times and metabolic half lives in the membrane which differ from the properties of the receptors at the adult neuromuscular junction (Berg & Hall, 1975;

Brenner & Sakmann, 1983). Coincidentally, the pharmacological properties of the Na channel change with the appearance of a new class of Na channels which are less sensitive to the blocking action of the neurotoxin tetrodotoxin (TTX) (Harris & Thesleff, 1971; Redfern & Thesleff, 1971a;b; Pappone, 1980; Lee, Miledi & Ruzzier, 1982; Rogart & Regan, 1985). Whether these new channels replace pre-existing Na channels is not clear.

In several respects, these changes in ACh receptors and Na channels, which are consequent to the denervation of adult skeletal muscle, are the reverse of those occurring during normal muscle development. Embryonic muscle has a high extrajunctional ACh receptor density (Diamond & Miledi, 1962; Fambrough & Rash, 1971; Bevan & Steinbach, 1977). These receptors have metabolic half lives and mean channel open times similar to those of adult extrajunctional receptors in denervated muscle (Sakmann & Brenner, 1978; Devreotes & Fambrough, 1975; Fischbach & Schuetze, 1980). With increasing age, ACh receptors become restricted to the endplate with transformation of their metabolic and channel properties (see 1.2). Similarly, mature Na channel numbers are very low in immature muscle but increase with age coincident with changes in their pharmacological properties (Sherman & Catterall, 1982; Lombet, Kazazoglou, Delpont, Renaud & Lazdunski, 1983; Harris & Marshall, 1973). The body of the evidence shows that these developmental changes are inhibited by

denervation (Brown, 1974; Vrbova, Gordon & Jones, 1978; Ziskind-Conhaim & Bennett, 1982; Sherman & Catterall, 1982; Schuetze & Vicini, 1984); indicating that these changes are dependent on the presence of an intact neural input. It has recently been shown that fluorescently labelled Na channels are localized to the synapses of innervated muscles and therefore the establishment of the adult gradient in Na channel density around the junction may also be dependent on neuronal influences (Angelides, 1986), although the continued presence of the nerve may not be required for the maintenace of this gradient (Caldwell & Milton, 1986). Thus the motoneuron plays a key role in the control of the numbers, distribution and properties of the muscle membrane proteins underlying the specialized muscle functions of chemosensitivity and excitability in both adult and developing muscle.

Several factors could be important in initiating the responses of adult muscle to denervation. These include: (1) the presence of the degenerating nerve terminals; (2) the decrease in muscle membrane excitation and/or contraction; (3) the absence of ACh or the loss of some other trophic factor(s) normally released by the nerve. Except for the degenerating nerve terminals, the other factors: activity; ACh and other trophic agents, may also be causally involved in the development of the adult pattern of numbers, distribution and properties of ACh receptors and Na channels.

Muscle paralysis or blockade of ACh, pre- or postsynaptically, induces supersensitivity, with increased incorporation of ACh receptors into the extrajunctional muscle membrane and a change in the TTX-sensitivity of the Na channels (Thesleff, 1960; Drachman, 1964; 1967; Berg & Hall, 1975; Tonge, 1974; Gordon & Vrbova, 1975; Pestronk, Drachman & Griffin, 1976; Cangiano, Lutzemberger & Nicotra, 1977; Lavoie, Collier & Tennenhouse, 1977; Mathers & Thesleff, 1978a;b; Bray, Hubbard & Mills, 1979; Sohal, Creazo & Oblak, 1979; Sellin & Thesleff, 1981; Brown, Hopkins & Keynes, 1982; Taxt, 1983). This suggests that neuromuscular activity (defined broadly as the release of ACh by the nerve and all of the subsequent postsynaptic events including ACh binding, membrane depolarization and muscle contraction) is important for the neuronal regulation of these muscle proteins. Reported differences between the magnitude of the changes produced by these treatments and a greater effect of denervation may be evidence for the presence of some other factor(s) besides neuromuscular activity in the mediation of nerve-muscle relations.

1.1.1 Questions. That denervation increases the numbers of ACh receptors in adult muscles is well established, but whether this reflects a dedifferentiation of the muscle membrane to a pre-innervation state is not known. The means by which the nerve regulates extrajunctional ACh receptor density and the extent to which neuromuscular

activity mediates this regulation are still not established. Further, the question of whether ACh receptors are similarly regulated in adult and in developing muscle is still open. The relative roles of ACh release as distinguished from muscle mechanical activity in regulating muscle properties remain to be elucidated and the potential effects of nerve degeneration products in the short-term modulation of denervation effects must also be considered.

With regard to the Na channel, it is still not clear whether denervation actually affects numbers of normal Na channels, although it definitely induces the appearance of an abnormal population. If denervation does decrease numbers of normal Na channels, are these co-regulated with ACh receptors and how is this regulation mediated after denervation of adult muscle and in developing muscle when Na channel numbers are rising as ACh receptor numbers fall?

The Na channel has several different toxin binding sites located on the intra- and extracellular faces of the channel protein. The presence of TTX-insensitive Na channels in immature and in denervated muscle suggests that the molecular composition of the channel protein may be changed. One way of addressing this question is to study the binding of the different neurotoxins to the channel and to ask whether these sites are present during development in their adult forms or whether there are

immature forms of the Na channel expressed during development. Because of the voltage-dependence of the binding of some classes of neurotoxins, these studies cannot be carried out in muscle homogenates but may be pursued using brain synaptosome preparations. The Na channel of skeletal muscle is very similar to the corresponding channel in other excitable tissues including central nervous system (CNS) neurons. Thus, information gathered from studies in brain may be applicable to the development of Na channels in muscle. In addition, if activity is important for the incorporation of mature Na channels in muscle, one can ask if it is also important for the expression of normal Na channel numbers in developing brain.

In the following sections, the current knowledge of the ACh receptor and the Na channel as well as their regulation by innervation will be reviewed. The specific questions addressed in the study will then be formulated and the approach taken in this work outlined.

1.2 The Acetylcholine Receptor

The nicotinic ACh receptor is a chemically-regulated ionophore and the best characterized neurotransmitter receptor (see reviews by Fambrough, 1979; Conti-Tronconi & Raftery, 1982; Popot & Changeux, 1984; McCarthy, Earnest, Young, Choe & Stroud, 1986). The receptor is an oligomeric protein which has been isolated from a range of tissues including the electric organs of Electrophorus electricus

(Meunier, Sealuck, Olsen & Changeux, 1974; Conti-Tronconi, Hunkapiller, Lindstrom & Raftery, 1982b), Torpedo californica (Lindstrom, Merlie & Yogeeswanan, 1979; Gullick & Lindstrom, 1982) and of Torpedo marmorata (Sobel, Weber & Changeux, 1977), as well as from piscine (Conti-Tronconi, Hunkapiller, Lindstrom & Raftery, 1984), bovine (Conti-Tronconi, Gotti, Hunkapiller & Raftery, 1982; Einarson, Gullick, Conti-Tronconi, Ellisman & Lindstrom, 1982; Gotti, Conti-Tronconi & Raftery, 1982) and cat (Shor, Dolly & Barnard, 1978) muscle. The functional identity of the purified protein has been confirmed in reconstitution experiments in which the pharmacological and electrophysiological responses of the in situ receptor were preserved when the purified protein was incorporated into lipid vesicles or planar bilayers (Anholt, Fredkin, Deerinch, Ellisman, Montal & Lindstrom, 1982; Changeux, Heidmann, Popot & Sobel, 1979; Heidmann, Sobel, Popot & Changeux, 1980; Lindstrom, Anholt, Einarson, Engel, Osame & Montal, 1980; Huganir & Racker, 1982; Criado, Eibl & Barrantes, 1984).

The ACh receptor is a pentameric protein composed of four different subunits, alpha,beta,gamma,delta, in the ratio 2:1:1:1. The molecular weights of the subunits are 38,000-44,000 (alpha), 48,000-53,000 (beta), 57,000-60,000 (gamma), and 64,000-67,000 (delta) daltons (Popot & Changeux, 1984). There is considerable homology between subunits as well as between ACh receptors from different

species (Conti-Tronconi, Hunkapiller, Lindstrom and Raftery, 1982a; Gullick and Lindstrom, 1982; Noda, Takahashi, Tanabe, Toyosato, Kikyotani, Furutani, Hirose, Takashima, Inayama, Miyata & Numa, 1983). All of the subunits for the Torpedo californica ACh receptor have been sequenced and cloned (Noda, Takahashi, Tanabe, Toyosato, Kikyotani, Furutani, Hirose, Takashima, Inayama, Miyata & Numa, 1982; Noda, Takahashi, Tanabe, Toyosato, Kikyotani, Hirose, Asai, Takashima, Inayama, Miyata & Numa, 1983; Claudio, Ballivet, Patrick & Heinemann, 1983) as have many of the subunits of the ACh receptor from the muscle of calf (Takai, Noda, Furutani, Takahashi, Notake, Shimuzu, Kayano, Tanabe, Tanaka, Hirose, Inayama & Numa, 1984; Tanabe, Noda, Furutani, Takai, Takahashi, Tanaka, Hirose, Inayama & Numa, 1984; Takai, Noda, Mishina, Shimizu, Furutani, Kayano, Ikedo, Kubo, Takahashi, Takahashi, Kuno & Numa, 1985), human (Noda, Furutani, Takahashi, Toyosato, Tanabe, Shimizu, Kikyotani, Kayano, Hirose, Inayama & Numa, 1983; Shibahara, Kubo, Perski, Takahashi, Noda & Numa, 1985) and mouse (LaPolla, Mixter Mayne & Davidson, 1984).

The five subunits of the ACh receptor are considered to be arranged symmetrically about a central pore. This hypothesis is supported by electron microscopic data (Brisson & Unwin, 1985). Each subunit crosses the plasma membrane five times with cytoplasmic COOH and extracellular NH_2 terminals (Lindstrom, Criado,

Hochschwender, Fox & Sarin, 1984; Young, Ralston, Blake, Ramachandra, Hall and Stroud, 1985). ACh binds to the alpha subunit of the receptor (McCormick & Atassi, 1984; Mishina, Takamasa, Imoto, Tanaka, Fujita, Fukuda, Kurasaki, Takahashi, Morimoto, Hirose, Inayama, Takahashi, Kuno & Numa, 1985) with two ACh molecules bound per receptor (Neubig, Boyd & Cohen, 1982). There are separate ACh binding sites associated with the activation and desensitization of the ACh receptor (Conti-Tronconi, Dunn & Raftery, 1982; Dunn & Raftery, 1982; Dunn, Conti-Tronconi & Raftery, 1983).

Although the exact mechanism of ACh receptor action is not known, studies with fast time resolution of the binding of ACh, coupled with electrophysiological studies of ACh receptor channel gating have increased our understanding of ACh receptor function. More recently, workers have begun to take advantage of the fact that Xenopus oocytes will express functional ACh receptors when injected with Torpedo or cat mRNA coding for the receptor (Sumikawa, Houghton, Emtage, Richards & Barnard, 1981; Miledi & Sumikawa, 1982) to explore the function of specified sequences of the molecule using the technique of site directed mutagenesis (Mishina et al., 1985).

As described above, the nicotinic ACh receptor is found in the electric organs of certain elasmobranches and electric fish and in skeletal muscle. Similar receptors are also found in brain and in autonomic ganglia although

these latter receptors are pharmacologically and immunologically somewhat different from the ACh receptor of skeletal muscle.

Study of the ACh receptor has been greatly facilitated by the existance of natural toxins with very high affinities for the receptor protein. Chief among them is alpha-bungarotoxin (BTX) isolated from the venom of the banded krait, Bungarus multicinctus (Chang & Lee, 1963; Dryden, Harvey & Marshall, 1974a;b). This toxin can be purified and iodinated to a high specific activity (Hartzell & Fambrough, 1972; Wang and Schmidt, 1980). BTX binds to the receptor with a stoichiometry of two toxins bound per receptor and has been frequently used to assay numbers of ACh receptors. The results obtained from binding assays agree well with parallel studies measuring ACh sensitivity directly (Hartzell & Fambrough, 1972; Land, Podleski, Salpeter & Salpeter, 1977). Of the two methods, the binding assays permit a more quantitative assessment of ACh receptor numbers. Using radio- or fluorescent-labelled BTX it has also been possible to measure the distribution and turnover of ACh receptors in the membrane (Berg & Hall, 1974; Fambrough, 1979; Peng, Cheng & Luther, 1981).

1.3 The Sodium Channel

The Na channel is a voltage-regulated ionophore which allows the inward flux of Na ions during the early, upward phase of the action potential. The channel is an integral

membrane protein with complex gating kinetics (Hodgkin & Huxley, 1952; Armstrong & Bezanilla, 1974 and reviewed in Hille, 1976) the study of which has been advanced by the more recent use of patch clamp recording of single channels (Sigworth & Neher, 1980; Sachs & Auerbach, 1983; Horn & Vandenberg, 1984).

The Na channel has been isolated from a variety of tissues including the Electrophorus electricus electric organ (Agnew, Levinson, Brabson & Raftery, 1978), rat brain (Hartshorne & Catterall, 1981; Messner & Catterall, 1985), heart (Lombet & Lazdunski, 1984) and skeletal muscle (Barchi, Cohen & Murphy, 1980). The purified protein has been reconstituted in liposomes and planar bilayers with preservation of its functional characteristics (reviewed in Barchi, Tanaka & Furman, 1984; see also Tamkun & Catterall, 1981; Villegas, Villegas & Suarez-Mata, 1981; Condrescu & Villegas, 1982; Talvenheimo, Tamkun & Catterall, 1982; Weigle & Barchi, 1982; Krueger, Worley & French, 1983; Moczydlowski, Garber & Miller, 1984; Rosenberg, Tomiko & Agnew, 1984; Tamkun, Talvenheimo & Catterall, 1984; Kraner, Tanaka & Barchi, 1985). The channel protein, as isolated from mammalian tissue appears to consist of two polypeptides, a 270 Kdalton alpha subunit and a 38 Kdalton beta subunit (Hartshorne & Catterall, 1981; Barchi et al., 1984). The functional significance of the beta subunit is unclear, since the active Na channel from electric organ consists

of only the large alpha chain (reviewed by Barchi et al., 1984; Catterall, 1986).

A wide variety of neurotoxins bind to the Na channel (reviewed by Catterall, 1979; Strichartz, 1981). The toxins include TTX and saxitoxin (STX) which bind to a site on the extracellular face of the channel and block Na flux (Catterall & Morrow, 1978; Strichartz, Rogart & Ritchie, 1979; Kao & Walker, 1982; Rubinson, 1982; Strichartz, 1984). Various scorpion toxins have been characterized which appear to bind to the inactivation gate in a voltage-dependent way (Narahashi, Shapiro, Deguchi, Scuka & Wang, 1972; Catterall, 1976b; Ovchinnikov & Grishin, 1982; Meves, Rubly & Watt, 1984), while the alkaloid toxins, veratridine and batrachotoxin, exert most of their effects by binding at the activation gate (Catterall, 1976a; Brown & Daly, 1981; Gola, Chagneux & Argemi, 1982; Gola, Ducreux, Chagneux & Argemi, 1982; Huang, Moran & Erhenstein, 1982).

The high affinity of the Na channel for these toxins permits their use in quantitative assays of Na channel numbers. Fluorescent (Angelides, 1981) and iodinated (Boudier, Berwald-Netter, Dellman, Boudier, Couraud, Koulakoff & Cau, 1985) derivatives of the toxins have also been used to study the localization of the Na channel. Monoclonal antibodies against the Na channel have now been raised (Moore, Fritz, Raftery & Brockes, 1982; Casadei, Gordon, Lampson, Schotland & Barchi, 1984; Meiri, Zeitoun,

Grunhager, Lev-Ram, Eshnar & Schlessinger, 1984). These antibodies are now being used to purify (Nakayama, Withy & Raftery, 1982) and localize (Haimovich, Bonilla, Casadei & Barchi, 1984; Mourne, Lombet & Lazdunski, 1984) the Na channel.

Na channels are widely distributed, being found in excitable tissues such as brain, heart and skeletal muscle, as well as in cultures of a number of non-excitable cells (Frelin, 1982; Chiu, Shrager & Ritchie, 1984; Shrager, Chiu & Ritchie, 1985), although whether the Na channels are present in the latter tissues in vivo is not clear. In skeletal muscle, Na channels are located both in the t-tubular membrane and in the sarcolemma (Jaimovich et al., 1976; 1983; 1986). In the sarcolemma, the channel density is graded with the greatest concentration near the neuromuscular junction (Beam et al., 1985). These Na channels are all functionally similar, although antigenic differences have been reported between Na channels in the nervous system and in skeletal muscle (Wollner & Catterall, 1985).

Channels at different developmental stages and in different tissues have different affinities for some of the neurotoxins (eg. Rogart, Regan, Dzienkan & Galper, 1983; Frelin, Vigne & Lazdunski, 1983; Frelin, Vijverberg, Romey, Vigne & Lazdunski, 1984). In particular, mammalian skeletal muscle in culture and embryonic muscles in vivo express Na channels which are only blocked by TTX at

concentrations 1000 times greater than for channels in innervated adult muscle. TTX or STX binding assays, which usually measure only high affinity sites, give information about the numbers of mature channels rather than of the total number of all Na channels types present. Labelled scorpion toxins are also used in binding assays but their voltage-dependence restricts their use to whole cell or synaptosomal preparations where the voltage difference across the membrane is preserved.

Structural studies of the Na channel have used various neurotoxins to map sites on the channel (Angelides & Nutter, 1983; Darbon & Angelides, 1984). The gene has been cloned and the primary structure has been determined for the Na channels of Electrophorus electricus (Noda, Shimizu, Tanabe, Takai, Kayano, Ikeda, Takahashi, Nakayama, Kanaoka, Minamino, Kangawa, Matsuo, Raftery, Hirose, Inayama, Hayashida, Miyata & Numa, 1984) and rat brain (Auld, Marshall, Goldin, Dawsett, Catterall, Davidson & Dunn, 1985). Recently functional Na channels have been induced in Xenopus oocytes (Hirono, Yamagashi, Ohara, Hisanaga, Nakayama & Sugiyama, 1985) thus paving the way for site-directed mutagenic studies to elucidate further the relations between the molecular structure and function of the Na channel.

1.4 Denervation of Adult Muscle

What effect does denervation have on mature muscle when the neuromuscular junction is formed and adult levels of

ACh receptors and Na channels are incorporated and maintained?

1.4.1 ACh receptors. Some of the muscle endplate specializations, including junctional folds and AChE accumulation, persist after denervation. Over long periods the endplate deposits of AChE, visualized histochemically, become amorphous and the junctional shape becomes less clear (Gwynn & Vrbova, 1966; Steinbach, 1981). The junctional ACh receptors maintain their normal channel open time (Brenner & Sakmann, 1983) while the pharmacology of the endplate receptors remains as before (Lorkovic & Rudel, 1984).

Extrajunctional ACh receptor density is very low in normal adult muscle. However, following nerve section, the ACh sensitivity of the denervated muscle greatly increases as a result of the incorporation of newly synthesized ACh receptors into the extrajunctional membrane (Kuffler, 1943; Reid & Williams. 1949; Axelsson & Thesleff, 1959; Miledi & Potter, 1971; Hartzell & Fambrough, 1972; Colquhoun et al., 1974; Brockes & Hall, 1975; Askmark et al., 1982a;b; Mong et al., 1982, Kallo & Steinhardt, 1983; Goldman et al., 1985). These new ACh receptors have the long channel open times and high rate of turnover in the membrane characteristic of neonatal extrajunctional ACh receptors (Brenner & Sakmann, 1983; Berg & Hall, 1974). The new ACh receptors are immunologically distinguishable from adult junctional receptors (Lefvert, 1982). Thus, in

terms of both the distribution and the type of ACh receptor, denervation of adult muscle appears to induce a dedifferentiation of the extrajunctional muscle membrane. It is not clear whether the numbers of ACh receptors which appear in the adult denervated extrajunctional muscle membrane become equivalent to the numbers in the extrajunctional membrane of immature skeletal muscle.

1.4.2 Na channels. There is no question that denervation alters some of the properties of the Na channel. However, the effects of denervation on Na channel numbers and distribution are less clear. Action potentials in denervated muscle become partially resistant to the effects of TTX, which normally blocks the inward Na current at nanomolar concentrations (Harris & Thesleff, 1971; Redfern & Thesleff, 1971a;b; Pappone, 1980; Lee et al., 1982). This TTX resistance is well developed within one week after nerve section, by which time up to 30% of the inward Na current can be blocked only by concentrations of TTX in the micromolar rather than the normal nanomolar range. Na channels in neonatal muscle are similarily TTX-resistant (Harris & Marshall, 1973; McArdle, Michelson & D'Alonzo, 1980).

Recently, both low and high affinity binding sites for ^{3}H-STX have been demonstrated in denervated muscle (Rogart & Regan, 1985), suggesting that the TTX-insensitive Na current is due to the appearance of Na channels with a lower affinity for the neurotoxin. These new channels thus

resemble the channels of neonatal muscle. Whether the appearance of these low affinity sites is accompanied by a decline in numbers of high affinity sites is still uncertain.

Quantitative assays of numbers of high affinity sites in denervated muscles have been carried out using the radiolabelled neurotoxins, ^{3}H-TTX (Colquhoun et al., 1974) and ^{3}H-STX (Ritchie & Rogart, 1977; Barchi & Weigle, 1979; Hansen-Bay & Strichartz, 1980). Reports using radioligand binding to whole muscle or to muscle homogenates have been equivocal but have generally concluded that denervation produced no significant decrease in the numbers of toxin binding sites (Ritchie & Rogart, 1977; Hansen-Bay & Strichartz, 1980). In contrast, a study using a highly purified sarcolemma preparation found a 40-50% decrease in toxin binding site density following denervation (Barchi & Weigle, 1979). Thus, it is not clear that denervation of adult muscle affects numbers of normal Na channels but channels of the neonatal type do appear to be present. Preliminary evidence suggests that the gradient of Na channel density reported for adult muscle (Beam et al., 1985) is preserved after denervation (Caldwell & Milton, 1986).

1.4.3 Muscle atrophy. Historically, one of the earliest reported consequences of muscle denervation is the loss of weight by the affected muscle. Denervation atrophy includes the loss of muscle wet weight (Gutmann and Young,

1944; Gutmann, 1948; Sola & Martin, 1953), muscle protein content (Metafora, Felsani, Cotrufo, Tajana, Di Iorio, Del Rio, DePrisco & Esposito, 1980) and a decrease in muscle fibre diameter (Pellegrino & Franzini, 1963; Engel & Stonnington, 1974; Cullen & Pluskal, 1977; Pachter, Eberstein & Goodgold, 1982; de Coster, De Reuck & Vander Eecken, 1985). This atrophy is a prominent feature of denervation in its own right and must also be considered in terms of its effects on measures of ACh receptors and Na channels, where radioligand binding is often expressed relative to muscle weight or muscle protein.

1.5 Muscle Development

Myogenesis and the subsequent differentiation of skeletal muscle are topics which have been widely studied. Myoblasts are derived from the mesoderm in embryonic development. During development, these myoblasts fuse to become multi-nucleated myotubes which then differentiate to form mature muscle fibres. The numbers of muscle fibres in rat diaphragm increase during gestation from 20% of adult numbers at day 16 *in utero* to 70% at birth. The remaining fibres are formed in the first postnatal week (Harris, 1981). Newborn fibres have been reported to be coupled by gap junctions (Schmalbruch, 1982). Rat muscle fibres are first innervated at day 14-15 *in utero* (Bennett & Pettigrew, 1974). In focally innervated muscle, each fibre develops one junctional area which initially receives input from several motoneurons (Redfern, 1970;

Bennett & Pettigrew, 1974; O'Brien, Ostberg & Vrbova, 1978). This polyneuronal innervation disappears in the first 2-3 weeks after birth until each fibre is contacted by only one motoneuron (Redfern, 1970; Bennett & Pettigrew, 1974; Brown, Jansen & Van Essen, 1976; O'Brien et al., 1978). During muscle development the adult patterns of ACh receptor and Na channel expression are established.

1.5.1 ACh receptors. Neuromuscular contact induces the formation of the new synapse with the development of complex junctional folds in the postsynaptic membrane, the specialization and deposition of the extracellular matrix (Zachs, 1964; Anderson, Klier & Tanguay, 1984; Bayne, Anderson & Fambrough, 1984; Anderson, 1986) and the accumulation of ACh receptors and AChE at the new muscle endplate (Tucek, 1972; Bennett & Pettigrew, 1974). The initial formation of the accumulations occurs at the site of nerve-muscle contact but, after a certain critical period, the patches persist in the absence of the nerve. Some early event at the new junction appears to encourage the aggregation of certain proteins. In this light it is of interest that certain adhesion molecules (Rutishauser, Grumet & Edelman, 1983; Anderson, 1986) become concentrated at neuromuscular junctions (Covault & Sanes, 1986). These adhesion molecules are glycoproteins and may also affect aggregation. The accumulation of ACh receptors at the junction may be due to interactions with these

adhesive macromolecules. Other factors could include homophilic interactions between receptors, perhaps potentiated by the nerve, and possibly some cytoskeletal involvement.

The ACh sensitivity of muscle increases during myogenesis and then, following innervation, extrajunctional ACh sensitivity declines and ACh receptors become concentrated at the motor endplate (Diamond & Miledi, 1962; Fambrough & Rash, 1971; Bevan & Steinbach, 1977). This decline in extrajunctional ACh receptor density begins soon after innervation. Numbers of receptors in rat muscle have been reported to drop to a plateau which is held through day 19 in utero to day 3 postpartum, followed by a second drop through the first 1-2 weeks after birth until the extrajunctional ACh receptor concentration is below $5/\mu m^2$ membrane surface area (Bevan & Steinbach, 1977). Just as denervation of adult muscle can increase numbers of extrajunctional ACh receptors, the normal develomental decrease in extrajunctional ACh receptor density does not occur if the muscle is denervated (Brown, 1974; Vrbova et al., 1978; Ziskind-Conhaim & Bennett, 1982).

Besides the above changes in the number and distribution of ACh receptors, the electrophysiological, pharmacological and immunological properties as well as the metabolic stability of the ACh receptor also change during development. Endplate ACh receptors of newborn rat

muscle fibres have mean channel open times of 4 ms. During the first 2 weeks of postnatal life, the mean channel open time decreases to the adult value of 1 ms (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980). A similar change has also been reported for Xenopus ACh receptors where endplates consist of a mixed population of receptors at an intermediate stage of development and, unlike rat muscle, the mean channel open time for the extrajunctional ACh receptors declines in parallel with the decrease for receptors at the junction (Kullberg, Brehm & Steinbach, 1981). The decrease in mean ACh receptor channel open time during development is inhibited by denervation (Schuetze & Vicini, 1984). Thus, during development, characteristics of both junctional and extrajunctional ACh receptors are under neuronal influence.

Curare is a competitive antagonist of ACh for the nicotinic receptor at the adult mammalian neuromuscular junction. However, for rat fetal and newborn muscle, both in vitro (Trautman, 1982) and in vivo (Ziskind & Dennis, 1978) curare depolarizes the muscle membrane, apparently by acting as a partial agonist at the ACh receptor. This response to curare begins to disappear between 0 and 3 days after birth and is gone after 5 days.

The antigenic characteristics of the receptor change postnatally as shown by the cross-reactivity with endplate receptors from newborn, 1 and 2 week old rat muscles of polyclonal antibodies specific for adult

extrajunctional, but not junctional, ACh receptors (Hall, Gorin, Silberstein & Bennett, 1985). Most of this cross-reactivity is gone after 2 weeks.

It had been reported that ACh receptors of immature muscle were degraded more rapidly than endplate ACh receptors in adult muscle, with half lives in the muscle membrane of 22-24 hrs as compared to 6 or more days, respectively (Devreotes & Fambrough, 1975). It now appears that this accelerated degradation rate is the property of only the extrajunctional ACh receptors and that ACh receptors at the junctions of young rats are as stable, metabolically, as those at adult endplates (Steinbach, Merlie, Heineman & Bloch, 1979). Of course, as the concentration of extrajunctional ACh receptors decreases during development, the average half-life of all the ACh receptors in the developing muscle membrane will be seen to increase. Considering this maintained stability of junctional ACh receptors, it is perhaps not surprising that the numbers of ACh receptors at the neonatal endplate continue to increase in the absence of innervation (Labovitz & Robbins, 1983).

1.5.2 Na channels. In contrast to the decreasing numbers of extrajunctional ACh receptors with maturation of the muscle, numbers of Na channels increase during muscle development. Experiments carried out on aneural muscle cultures demonstrate the rise in Na channel numbers with development in rat (Frelin et al., 1983; Sherman,

Lawrence, Messner, Jacoby & Catterall, 1983; Frelin et al. 1984) or chicken (Catterall, 1980; Frelin, Lombet, Vigne, Romey & Lazdunski, 1981; Baumgold, Parent & Spector, 1983a;b; Strichartz, Bar-Sagi & Prives, 1983). In the postnatal development of rat muscle *in vivo*, high affinity STX or TTX binding site numbers are initially very low (Sherman & Catterall, 1982; Lombet et al., 1983) rising to plateau values by 3 weeks after birth.

There appears to be an immature form of the Na channel which binds scorpion toxin but not STX or TTX (Baumgold et al., 1983a; Frelin et al., 1983; Strichartz et al., 1983) Studies on rat muscle in culture which compared the binding of different toxins to the Na channel and the effect of those toxins on ^{22}Na flux revealed several different forms of the Na channel during development. The earliest appearing Na channels in *in vitro* development are not activated by voltage, but a ^{22}Na flux may be stimulated by the alkaloid toxins, batrachotoxin and veratridine, which open the activation gate of the Na channel. This flux is potentiated by the addition of scorpion toxin to inhibit inactivation. With increasing time in culture, these channels become voltage-gated but remain more resistant than normal Na channels to the blocking action of TTX (Frelin et al., 1984). Early TTX-resistant Na channels appear to have high affinity binding sites for scorpion toxins but not for STX (Baumgold et al., 1983a). In contrast to chick muscle in culture, the

^{22}Na flux in cultured rat skeletal muscle never develops a higher sensitivity to TTX (nanomolar as compared with micromolar) (Frelin et al., 1984).

Interestingly, in the *in vitro* development of rat muscle, high affinity TTX binding is measurable when Na channels are not sensitive to the blocking action of the toxin. If similar binding sites, apparently unrelated to functional Na channels, are present during *in vivo* muscle development, it would vitiate the use of radiolabelled TTX or STX binding for the measurement of Na channel incorporation in maturing muscle membranes. The aquisition of TTX sensitivity by newborn rat muscle fibres by the third week postpartum (Harris & Marshall, 1973) coincides with the time of appearance of high affinity TTX or STX binding (Sherman & Catterall, 1982; Lombet et al., 1983), so that it would appear that TTX-sensitive channels with high affinities for TTX and STX replace the immature TTX-insensitive channels. Thus, contrary to the situation *in vitro*, neonatal muscles *in vivo* do not express high affinity TTX binding sites which are unrelated to functional Na channels.

The TTX-insensitive Na channels of immature muscle may be related to the TTX-insensitive Na channels of denervated adult muscle. Innervation appears to be required for the development of TTX-sensitivity since embryonic rat muscle explants, taken after the muscle has been first innervated and subsequently raised in aneural

cultures, do not develop Na currents that can be blocked by nanomolar concentrations of TTX (Frelin et al., 1984). With regard to the maturational increase in the numbers of high affinity ^{3}H-STX or TTX sites (Sherman & Catterall, 1982; Lombet et al., 1983), denervation at 5, 11 or 17 days after birth reduces the maximum numbers of ^{3}H-STX binding sites to 50% of normal adult values (Sherman & Catterall, 1982). Postnatal incorporation of mature Na channels would thus appear to be modified by, but not totally dependent on, innervation. This would agree with the effects of denervation of adult muscle where some, but not all, of the Na channels become TTX-insensitive (Pappone, 1980).

1.6 Development of the Na channel in mammalian brain

Na channel incorporation into nervous tissue has been studied *in vitro* (Berwald-Netter, Couraud, Koulakoff & Martin-Moutot, 1982; Reiser, Loffler & Hamprecht, 1983; Couraud, Martin-Moutot, Koulakoff & Berwald-Netter, 1986) and *in vivo* (DeVries & Lazdunski, 1982; Baumgold, Zimmerman & Bambrick, 1983; Buonanno & Villegas, 1983; Baumgold, 1985). Adult Na channel density is much higher in brain than in muscle (of the order of 1000 versus 50 fmol/mg protein, compare Baumgold et al., 1983; Bambrick & Gordon, 1986b). The developmental time course of Na channel incorporation is very similar in rat brain and muscle with the numbers of high affinity ^{3}H-STX binding sites being very low at birth and rising to adult levels

by 3 weeks postpartum (Baumgold et al., 1983) despite the differences between the environments of the two cell types. The binding of the voltage-dependent scorpion toxins can be measured in synaptosomal preparations from brain as a voltage differential is maintained across the synaptosomal membrane. It has been reported that, as for muscle cells in culture, scorpion toxin binding in developing rat brain in vivo reaches adult values before the appearance and attainment of the adult plateau numbers of STX binding sites (Baumgold, 1985). This corroborates the finding that immature form(s) of the Na channel appear during the in vivo development of excitable tissue. The relationships between the development of Na channels in brain, synaptogenesis and neuronal activity are not clear.

1.7 Mechanisms of neuronal regulation of muscle ACh receptors and Na channels

Extrajunctional ACh receptors and Na channels are both seen to be regulated by innervation in developing and adult muscle, but it is not clear how this regulation is mediated.

Nerve section deprives the muscle of all contact with the nerve. Denervated muscle is no longer exposed to ACh, the membrane is not electrically excited and the muscle fibres no longer contract. Any of these changes may contribute to the regulation of muscle ACh receptors and Na channels. Many workers have designed experiments to study each of these changes in isolation in order to

assess their importance in neuronal regulation of muscle properties.

1.7.1 The role of muscle activity. If muscle activity is important for the regulation of muscle ACh receptors and Na channels, then inactivity should result in elevated extrajunctional ACh receptors and the emergence of TTX-insensitive action potentials. In addition, electrical stimulation of denervated muscle should retard the development of ACh supersensitivity and changes in Na channel properties.

1.7.1.1 Adult muscle. Muscle atrophy ensued when the hindlimbs of experimental adult animals were immobilized by braces (Edgerton, Barnard, Peter, Maier & Simpson, 1975), plaster casts (Witzman, Kim & Fitz, 1982), joint fixation (St-Pierre & Gardiner, 1985) or subjected to suspension-induced disuse (Gupta, Misulis & Dettbarn, 1985). In no case were the effects of immobilization as pronounced as the changes consequent to denervation. An exact comparison with the denervated state was difficult, however, because the electromyographic (EMG) activity was not completely abolished in these models (see also Fournier, Roy, Perham, Simard & Edgerton, 1983, for a critique of limb immobilization techniques).

Limb immobilization studies are, therefore, unsatisfactory as models of inactivity since some EMG activity can still be recorded in the immobilized muscles. Isolation of cat motoneurons from all central or sensory

input by spinal cord section and rhizotomy produces a complete flaccid paralysis of the affected muscle with no EMG activity recorded for periods of up to 2-3 years after the surgery (Tower, 1937; Eldridge, Liebhold & Steinbach, 1981; Eldridge, 1984b). The muscle fibres of these isolated motor units atrophy and the distribution of ACh receptors in the muscles is abnormal, although it does not show the extensive spread of ACh receptors to the extrajunctional region found in denervated muscles (Eldridge et al., 1981).

The induction of denervation-like changes in muscle fibres by isolation of the motor unit supports a role for activity in the neuronal regulation of muscle properties. The reported differences between the degree of muscle atrophy and disturbances in ACh receptor distribution in denervated as opposed to quiescent muscles, suggests that some other factor may also be exerting an important regulatory influence.

Another approach to modifying muscle activity while preserving the motor innervation intact has been to block nerve impulse conduction by pressure block (Gilliatt, Westgaard & Williams, 1977), local anaesthetics (Lomo & Rosenthal, 1972; Cangiano et al., 1977; Mong et al., 1982) or the Na channel antagonist, TTX (Pestronk et al., 1976; Lavoie et al., 1977; Bray et al., 1979; Taxt, 1983) In each case, the neuromuscular connections were functional since muscle excitation was still evoked by stimulation of

the nerve distal to the site of mechanical or chemical block. The effect of the impulse blockade in these experiments was to decrease muscle resting membrane potential, to induce the appearance of both fibrillation potentials and TTX-insensitive action potentials (Cangiano et al., 1977) and to increase the ACh sensitivity (Gilliatt et al., 1977) and the numbers of ^{125}I-BTX binding sites (Pestronk et al., 1976; Lavoie et al., 1977) of the affected muscles. All of these effects are qualitatively similar to the changes which occur in denervated muscle. Lomo & Rosenthal (1972), in fact, reported that nerve impulse blockade and denervation induced an equivalent increase in muscle chemosensitivity. One of the difficulties in the use of local anaesthetics to block nerve impulse conduction, however, was that degeneration of the nerve often ensued and many of the muscle fibres supposed to be only inactive may have been denervated (Blunt & Vrbova, 1975; Lavoie et al., 1977). A better blockade of impulse traffic with no adverse effects on the nerve was achieved using the highly potent neurotoxin TTX (Pestronk et al., 1976; Lavoie et al., 1977). In these experiments, the increase in chemosensitivity or ^{125}I-BTX binding sites did not equal the effects of nerve section. In adult muscle, therefore, muscle inactivity due to either restraint or the inhibition of nerve impulse activity only partially mimicked denervation.

Electrical stimulation of denervated muscles has been shown to retard denervation changes, showing that muscle activity can replace the trophic influence normally supplied by the nerve. The increase in ACh sensitivity (Lomo & Westgaard, 1975) or ^{125}I-BTX binding (Linden & Fambrough, 1979) normally occurring after denervation of adult rat muscle could be reduced or even abolished for periods of up to 3 weeks by stimulation. The same was true for the post-denervation changes in contractile properties (Lomo, Westgaard & Engelbretsen, 1980) and resting membrane potential (Lomo & Westgaard, 1975).

1.7.1.2 Developing muscle. The effects of limb immobilization or nerve conduction block on muscle properties have not been extensively studied in developing animals. The importance of activity in the development of embryonic rat muscles was studied by co-culturing neurons with muscle cells in the presence or absence of sufficient TTX to block all electrical activity in both nerve and muscle cells. The muscles apparently developed normally without activity. Primary clusters of ACh receptors formed at inactive neuromuscular junctions but the aquisition and maintenance of junctional AChE as well as the elimination of extrajunctional ACh receptor clusters only occured in TTX free cultures (Ziskind-Conhaim & Bennett, 1982). By comparisons with aneural muscle cultures, the authors concluded that nerve contact was sufficient to initiate endplate specialization by the

muscle but that the nerve regulated the extrajunctional ACh receptor population by electrical activity (Ziskind-Conhaim & Bennett, 1982).

Electrical stimulation promotes growth and survival and decreases ACh sensitivity in aneural chick muscles (Cohen & Fischbach, 1973; Bloom, Butler, Brierly & Cosmos, 1985) and restores the normal ACh sensitivity in rabbit muscles denervated at birth (Brown, 1974). Therefore, electrical stimulation can, at least partially, substitute for the trophic action of the nerve in mature and developing muscles.

1.7.2 The Role of Acetylcholine

Reported differences between the effects of nerve impulse blockade and a greater effect of denervation (see 1.7.1, above) on muscle properties suggest that the nerve may exert a trophic effect on the muscle in the absence of evoked ACh release and muscle contractile activity. Thesleff (1960) observed an inverse correlation between miniature endplate potential (mepp) frequency and muscle ACh sensitivity at neuromuscular junctions where presynaptic release of ACh was inhibited by botulinum toxin (BoTX). He concluded that the spontaneous, non-evoked release of ACh was the trophic factor.

At the motor nerve terminal, nerve impulses evoke the quantal release of ACh (del Castillo & Katz, 1954; Katz & Miledi, 1967; Hancox & Scrimshire, 1982). In the absence of nerve impulses, some ACh is released spontaneously. A

portion of this non-evoked release of ACh is in the form of quanta, which give rise to mepp's at the postsynaptic membrane (Fatt & Katz, 1952). A larger fraction of the spontaneous release of ACh is non-quantal (Fletcher & Forrester, 1975; Katz & Miledi, 1977).

Blockade of ACh action, either by inhibiting its release (presynaptic blockade) or by interfering with its interaction with the ACh receptor (postsynaptic blockade) has been shown to have denervation-like effects on the chemosensitivity and electrical excitabilty of adult and developing muscle.

In adult animals, postsynaptic blockade of ACh with d-tubocurarine (dTC) or BTX was shown to be equivalent to denervation in increasing extrajunctional ACh sensitivity (Berg & Hall, 1975; Lavoie et al., 1977; Chang, Chuang & Huang, 1975). Blockade of ACh release with BoTX has been shown to induce increased ACh sensitivity in the affected muscle (Thesleff, 1960; Tonge, 1974; Pestronk et al., 1976; Mathers & Thesleff, 1978; Sellin & Thesleff, 1981; Brown, et al., 1982). This is accompanied by a decline in the rate of rise of the action potential and the appearance of TTX-insensitive Na currents (Mathers and Thesleff, 1978). BoTX acts from the inside of the presynaptic terminal and it has been suggested that it acts by reducing the Ca sensitivity of the ACh release mechanism (Cull-Candy, Lundh & Thesleff, 1976). Ca influx itself is unaffected (Dreyer, Mallart & Brigant, 1983).

Impulses invade the terminals as usual and endplates appear structurally normal (Drachman, 1964; Duchen, 1971a; Harris & Miledi, 1971).

Neuromuscular blockade of avian muscles developing *in ovo* by pre- and postsynaptic blockade of neuromuscular transmission with hemicholinium-3 (Gordon & Vrbova, 1975) or BoTX (Drachman, 1964; 1967) and dTC (Gordon & Vrbova, 1975) or BTX (Sohal et al., 1979) respectively, results in muscle atrophy, inhibits the proper development of innervation and prevents the decline in muscle chemosensitivity that normally follows innervation. Presynaptic blockade of neonatal rat muscles with BoTX delays the elimination of polyneuronal innervation (Brown, Holland & Hopkins, 1980) as does postsynaptic blockade (Duxson, 1982).

The denervation-like effects induced by pre- or postsynaptic blockade of ACh do not necessarily argue for a specific trophic role of ACh since the effects of these drugs may be due to inactivity produced in the treated muscles. However, in some experiments where the effects produced by BoTX always fell short of denervation, the addition of alpha-neurotoxin (a postsynaptic blocker of ACh) to BoTX-poisoned muscles increased the magnitude of the observed changes to denervation levels (Mathers & Thesleff, 1978). These results have been interpreted as demonstrating the importance of non-evoked ACh release in the neuronal regulation of muscle properties. This

explanation was put forward on the grounds that BoTX blocks evoked but not all the non-evoked release of ACh from the nerve terminals.

The magnitude of the non-evoked release of ACh remaining in the presence of BoTX is still a matter of controversy. Stanley & Drachman (1983) showed that BoTX blocks quantal, but not non-quantal, release of ACh at the junction. Dolezal, Vyskocil & Tucek (1983), using the same method as Stanley & Drachman, 1983, found an almost 40% decrease in non-quantal spontaneous release from BoTX poisoned nerves. Gas chromatographic assays of ACh also demonstrated a decrease in spontaneous ACh release from BoTX-poisoned preparations that showed a partial inhibition of the non-quantal component of spontaneous release (Gunderson & Jenden, 1983).

Along with the disagreement as to magnitude of the non-evoked ACh release which is blocked by BoTX, some studies have found, in contrast to the results reported above, equivalent effects of BoTX and denervation on ACh sensitivity and other atrophic changes in adult mouse muscle (Brown et al., 1982) and in developing chick muscle (Drachman, 1967). Possibly, as was suggested originally by Thesleff (1960), BoTX may have a graded effect on muscle properties which is dependent on the degree of blockade of non-evoked ACh release in the preparation. If the spontaneous release of ACh is a trophic factor for muscle, evidence from the equivalent effects of ACh receptor

blockade and denervation (Lavoie et al., 1977; Mathers & Thesleff, 1978) would suggest that this action of ACh is mediated via an interaction with the nicotinic ACh receptor.

1.7.3 Role of nerve breakdown products.

Several studies of the reversibility of the effects of denervation by electrical stimulation of the muscle have shown that there was a 2-3 day period when stimulation was not effective in preventing the development of ACh supersensitivity by the muscle. This coincided with the time when the nerve terminal was degenerating, suggesting that products of nerve terminal breakdown may contribute to the development of muscle ACh supersensitivity (Jones & Vrbova, 1974; Lomo & Westgaard, 1975). The ability of such products to induce local muscle supersensitivity was verified in experiments where nerve segments were implanted on the surface of innervated muscles (Vrbova, 1967; Jones & Vrbova, 1974; Jones & Vyskocil, 1975). The effect of the degenerating nerve was to induce local accumulations of ACh receptors. This effect was temporary if the muscle was active, indicating that the breakdown products could induce but not maintain muscle supersensitivity (Lomo & Westgaard, 1975).

The degenerating nerve terminal may be involved in early muscle changes after denervation, but it is not clear how this relates to either the inactivity-induced increase in chemosensitivity in adult muscle or to the

presence of ACh receptors in the extrajunctional regions of neonatal muscle.

1.7.4 Role of other trophic substances.

The inequality between the effects of degeneration and those of nerve impulse blockade on muscle properties has also been considered as evidence that the motoneuron secretes some non-ACh trophic factor. Studies of the effects of nerve extracts (Podleski, Axelrod, Ravdin, Greenberg, Johnson & Salpeter, 1978; Jessell, Siegel & Fischbach, 1979) or medium from cultured neurons on muscle cells in vitro (Schaffner & Daniels, 1982) also suggest the existence of some trophic factor. Many of these experiments measured the ability of various putative trophic substances to induce ACh receptor clumping. These factors may be important for the establishment of the neuromuscular junction but do not, to date, appear to be major factors in the neuronal regulation of extrajunctional ACh receptors or of Na channels.

1.8 Proposal.

Several questions on the neuronal regulation of muscle Na channels and ACh receptors have been addressed in this study using radioligands to quantify the levels of ACh receptors and Na channels in developing and mature mammalian skeletal muscle and brain. Numbers of ACh receptors and Na channels in denervated adult and immature muscle have been compared in order to assess the extent to which denervation may be described as inducing

dedifferentiation (Chapters 3 & 5). The role of neuromuscular activity in mediating the neuronal regulation of these two proteins has been clarified by comparing the effects of nerve section with those consequent to the presynaptic blockade of neuromuscular transmission with BoTX in mature and developing muscle (Chapters 3 & 5). The local and systemic toxicity of BoTX was studied in adult and immature rats (Chapter 8). The long term effects of denervation and the effects of loss of muscular activity, as compared to the inhibition of ACh release, have been evaluated in studies of denervated cat muscles and cat muscles rendered quiescent by spinal isolation of their motoneurons (Chapter 4). The incorporation of Na channels in developing muscle and brain was compared and the development of the mature Na channel was studied in brain with the measurement of the increase in the binding of two different Na channel toxins with age (Chapter 7).

CHAPTER TWO: METHODS

2.1 General Methods

ACh receptors and Na channels were assayed in adult and developing rat hind limb muscles. The effects of nerve section and BoTX-paralysis were compared to assess the role of neuromuscular activity in the neuronal regulation of these two membrane proteins. ACh receptor numbers were also assayed and compared between denervated and quiescent cat hindlimb muscles to clarify further the importance of muscle activity in this regulation. Na channels were studied in developing rat brain. Toxins binding to two separate sites on the channels were compared permitting a in order to consider the maturation of the Na channel protein itself.

Denervated, BoTX-treated and control triceps surae (minus soleus; t.s.) muscles from treated and untreated Sprague Dawley rats of various ages were homogenized in ice cold buffers. Similar homogenates were prepared from cat hindlimb muscles which had been denervated by ventral root section or inactivated by spinal isolation of the motor unit. ACh receptors and Na channel numbers were assayed in the homogenates using ^{125}I-BTX and ^{3}H-STX, respectively, in rapid filtration assays. Rat weights, muscle weights and muscle fibre diameters were also measured. Using similar radioligand binding techniques, ^{3}H-STX and iodinated scorpion toxin (^{125}I-ScTX) binding to the Na channels of developing and adult rat brain was also

assayed.

2.2 Research animals: source and experimental treatment.

2.2.1 Animal source. Female adult Sprague Dawley rats were obtained from the Biosciences breeding colony at the University of Alberta. Neonatal rats came either from this colony or from Charles River Inc., Quebec. Fetal Sprague Dawley rats were obtained by Caesarian section of timed pregnant females from Charles River Inc. Male and female neonatal and fetal rats were used. Some of the studies of Na channels in developing rat brain were done at the National Institute of Health in Bethesda Maryland. These rats were Sprague Dawley rats from Seconic Farms, U.S.A. The domestic cats (male and female) used in the inactivity experiments, with one exception (from the University of Alberta), came from and were operated on at, the University of California at Los Angeles.

2.2.2 Sciatic nerve section. Hindlimb muscles of adult or neonatal Sprague Dawley rats were denervated under halothane anesthesia and aseptic conditions by the excision of a 5-10 mm segment from the sciatic nerve uni- or bilaterally. The excision was made 1 cm above the knee, before the division of the sciatic into the tibial and peroneal nerves (Fig. 2.1). The success of the operation was assessed as described below for BoTX-treated muscles. In addition, visual inspection, at the time the muscles were removed, revealed the continued presence of a discontinuity in the sciatic nerve. Muscle reinnervation

did not occur over the time course of this study.

2.2.3 BoTX-treatment. BoTX was administered in a minimum volume as a subcutaneous injection into one hindlimb just below the knee (Sellin & Thesleff, 1981; Brown et al., 1982). Except where otherwise noted, adult rats received 36 ng BoTX per kg rat weight in a volume of less than 25 µl. This was equivalent to the dosage used by Brown et al. (1982) where mice received a subcutaneous injection into the lower hind limb equivalent to 10 intraperitoneal LD_{50} doses. In this way neuromuscular transmission in the hindlimb could be effectively blocked for up to 2 weeks, although 11 days was usually the limit for complete block.

Neonatal rats (1-3 weeks postpartum) received doses varying from 14 to 58 ng BoTX per kg rat weight. BoTX blockade of neuromuscular transmission proved to be very difficult to maintain in immature rats without the use of doses so high as to cause extreme weight loss. As a result, neuromuscular transmission in neonatal rats could only be completely blocked for 3-4 days and incompletely blocked for 6-8 days. BoTX toxicity in adult and neonatal rats is discussed in Chapter 8.

The effectiveness of the BoTX blockade was monitored by loss of the toe spread reflex. When the animal is suspended in air, supported by the experimentor's hand around the chest just below the fore limbs, and rapidly lowered without being dropped, spreading of the toes is reflexively elicited. This reflex is lost after

interruption of the peroneal nerve and has been used in previous studies as evidence for continued denervation (Gutmann, 1948). This reflex was lost after section of the sciatic nerve or neuromuscular blockade as was reflexive foot-withdrawal in response to toe pinch. The effectiveness of the treatment was further assessed in anesthetized rats prior to removal of the muscles by suprathreshold stimulation of the sciatic nerve. BoTX-treated rats in which any toe spreading or foot withdrawal occurred or where indirect stimulation elicited muscle contraction were considered as incompletely blocked.

2.2.4 Control rat muscles Untreated hindlimb muscles from age-matched animals were used as controls. Innervated muscles contralateral to the denervated muscles were sometimes used as controls but never muscles contralateral to BoTX-treated hindlimbs, as the BoTX may enter the systemic circulation and may, therefore, impair neuromuscular transmission in the opposite side (Bambrick, Gordon & Baumgold, 1983).

2.2.5 Cat root section, spinal isolation and sciatic nerve section. All root section and spinal isolation operations were performed by Dr. L. Eldridge, Department of Physiology, University of California, Los Angeles. The surgery involved the lumbrosacral innervation from spinal segments L5-S3 which serves the lower hindlimbs. The cats were anaesthetized with pentobarbital and the surgery was carried out under sterile conditions.

For section of cat ventral roots, the lower hindlimb muscles were completely denervated by the section of ventral roots L5, L6, L7, S1 and S2. To insure that no regeneration occurred, a portion of the cord was removed.

The spinal isolation operation, surgery and care, has been described previously in detail (Eldridge, 1984b). Briefly, the spinal cord was transected between spinal segments L4 and L5 and again just rostral to spinous process S1 (Fig. 2.2). This effectively isolated spinal segments L5-S2 or S3 (Eldridge, 1984a). A complete dorsal rhizectomy was carried out in the entire region between the two transections. Great care was taken to preserve the ventral artery intact. This procedure resulted in complete flaccid paralysis of the lower hindlimbs persisting throughout the time course of this study with no motoneuron loss (Liebhold & Eldridge, 1981; Eldridge, 1984b).

Electromyogram (EMG) activity in hindlimb muscles (medial gastrocnemius and tibialis anterior) of operated cats was intermittently monitored every 2-4 days for the first 3 weeks after surgery and then at progressively longer intervals for the next 7 months. In all EMG testing the cats were awake and unrestrained in the investigator's lap during the recording. EMG, as recorded for several minutes using 3 straight stainless-steel needle electrodes, teflon-coated except at the tip, attached to a Neurodiagnostics 1-K electromyograph with X500

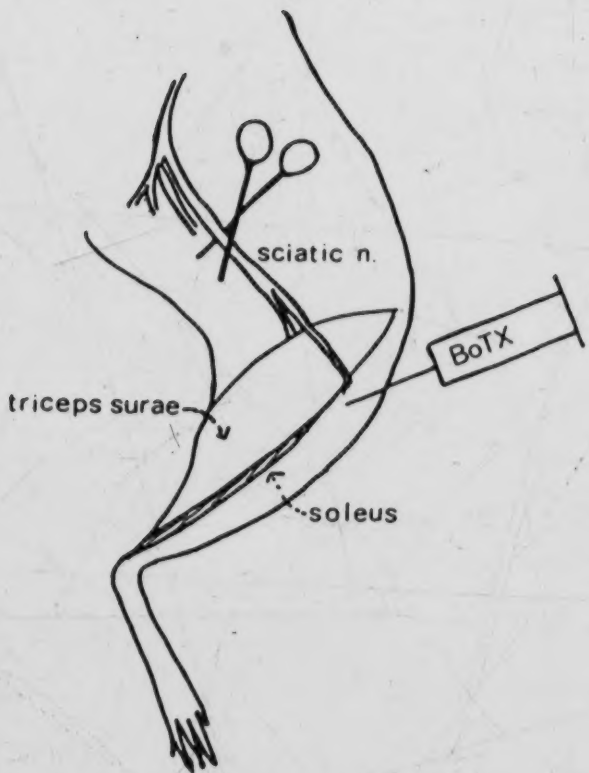


Fig. 2.1. Rat hindlimb muscles were denervated by sciatic nerve section or paralysed by BoTX injection.

amplification (Eldridge, Dhoot & Mommaerts, 1984), provided an indication of any recovery of function by the affected muscles as well a monitor of the occurrence of positive sharp waves, irritability and fibrillation potentials in the muscles (Eldridge & Mommaerts, 1984). In addition sensory stimulation of the hindlimbs, including passive muscle extension, tactile or noxious stimulation was used to monitor the effectiveness of the spinal cord section and rhizotomy.

In one cat, the right lower hindlimb muscles were denervated by section of the sciatic nerve and excision of a 1-2 cm segment of the sciatic. This procedure was carried out in this laboratory using pentobarbital anaesthesia and sterile procedure. No reinnervation was observed over the 2 months before the acute experiment or post-mortem.

2.3 Preparation and quantification of radioligands and toxins.

2.3.1 Bungarotoxin. ^{125}I-BTX used had a specific activity of >200 Ci/mmole and a radiochemical purity of >98% (Amersham Canada Limited). Unlabelled BTX was purchased from Sigma Chemical Company.

2.3.2 Saxitoxin and Tetrodotoxin. Unlabelled STX was obtained from the American Food and Drug Administration or purchased from Calbiochem, Behring Diagnostics, U.S.A. Tetrodotoxin was obtained from Sigma Chemical Company. Unlabelled STX was tritiated by Amersham Limited following

the method of Ritchie, Rogart & Strichartz (1976). Subsequently the ^{3}H-STX was purified using high voltage electrophoresis either by Dr. J. Baumgold at the NIH, Bethesda, Md or by the author at this university. In both cases the procedure used was a modification of Ritchie et al. (1976).

In Bethesda, the ^{3}H-STX was dissolved in 0.1 M (2-[N-Morphilino]ethane)sulfonic acid, sodium salt (MES), pH 5.0. This solution was subjected to electrophoresis on paper for 60 minutes at 50-60 V/cm using a solution of pyridine:MES:water, 25:1:225 at pH 6.5. By use of a cooling plate the temperature was maintained at 2-4 °C throughout the electrophoretic run. The paper was then partially dried at 4 °C and cut into 1 cm strips. Each strip was eluted with 2 mls 0.1 M MES-NaOH pH 5.0. After mixing each strip with buffer, a 5 µl aliquot was counted. Plotting cpm vs fraction number yields a chromatogram with three major peaks (Fig. 2.3A). All of the biological STX activity resides in the third peak. When the procedure is repeated for a small sample of the fractions from peak 3 the eluant was found to migrate as a single peak.

For ^{3}H-STX purification at this University, the above procedure was modified by using thin layer cellulose coated plates and a portable electrophoresis unit which could be kept in the cold room to maintain the temperature below 5 °C. An aliquot of the impure tritiated toxin was

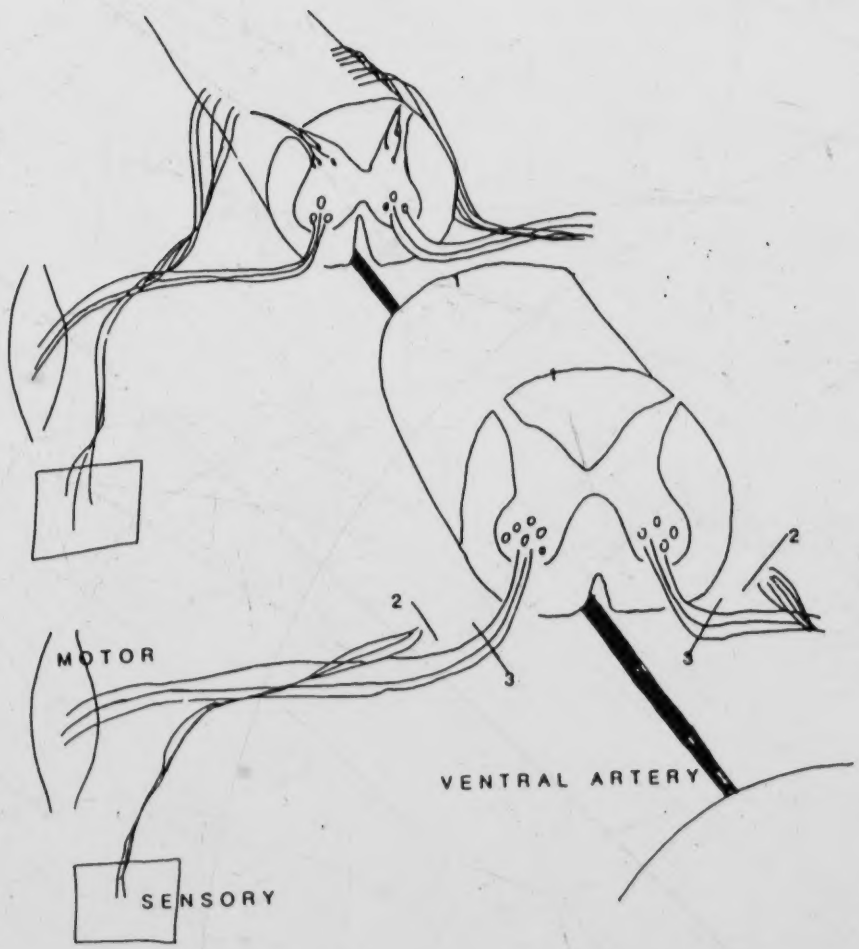


Fig. 2.2. Diagramatic representation of cat spinal cord showing sites of: 1) cord transection for spinal isolation; 2) dorsal rhizotomy; 3) ventral root section for muscle denervation.

dissolved in a small volume of 0.1 M sodium-acetate, pH 5.0 (Ritchie et al. 1976). This solution was subjected to electrophoresis at 20 V/cm across the thin layer cellulose plate. The electrophoretic run was done with a solution of pyridine:acetic acid:water, 33:1:299 at pH 6.5 for 120 minutes. The plate was air dried in the cold room and then scored into 0.5 cm sections. Each section was scraped into a glass vial and shaken with 2-5 mls of 0.1 M sodium acetate pH 5.0 at 4°C. Five µl aliquots were taken and counted and the cpm plotted vs the distance from the origin (Fig. 2.3B). As was described earlier, all of the biological activity was in the third fraction. This was determined by assaying pooled fractions from each peak for TTX-displacable ^{3}H-STX binding to a rat brain homogenate preparation.

2.3.3 ^{3}H-STX: concentration and radiochemical purity The ^{3}H-STX concentration and radiochemical purity were determined with two methods which gave similar results. In Dr. Baumgold's laboratory at the NIH, Bethesda, Maryland, classical pharmacological techniques were used in a bioassay measuring the effect of STX in blocking the regenerative action potential. In this laboratory, the STX inhibitable binding of the ^{3}H-STX was used to determine the concentration of the tritiated toxin.

The ^{3}H-STX concentration was established by Dr. Baumgold in his laboratory using the desheathed frog sciatic nerve preparation (Ritchie et al., 1976). Briefly,

a desheathed frog sciatic nerve was mounted in a chamber equipped with a pair of stimulating and recording electrodes. The nerve was exposed to known concentrations of STX for 5 minutes and the decline in the compound action potential measured and plotted against drug concentration to obtain a standard curve. The toxin was washed off the nerve for 7 minutes before the next sample was applied. The reduction in the action potential amplitude was then determined for several doses of the purified ^{3}H-STX and its concentration estimated (Fig. 2.4).

The radiochemical purity was determined by incubating the ^{3}H-STX with desheathed rabbit vagus nerves and computing the fraction of total radioactivity and of total biological activity taken up by the nerves. Since the toxin is not radiochemically pure, relatively more biological activity than radioactivity will be taken up by the nerves. After subtraction of the nonspecific uptake, measured in the presence of a large excess of TTX, the ratio of the fraction of biological activity to the fraction of radioactivity bound can be used to determine the radiochemical purity. In one such experiment the apparent specific activity was 24 Ci/mmole and the radiochemical purity was 50%. Since only 50% of the tritium was associated with the STX, the true specific activity was 12 Ci/mmole.

Quantification of the ^{3}H-STX in this laboratory

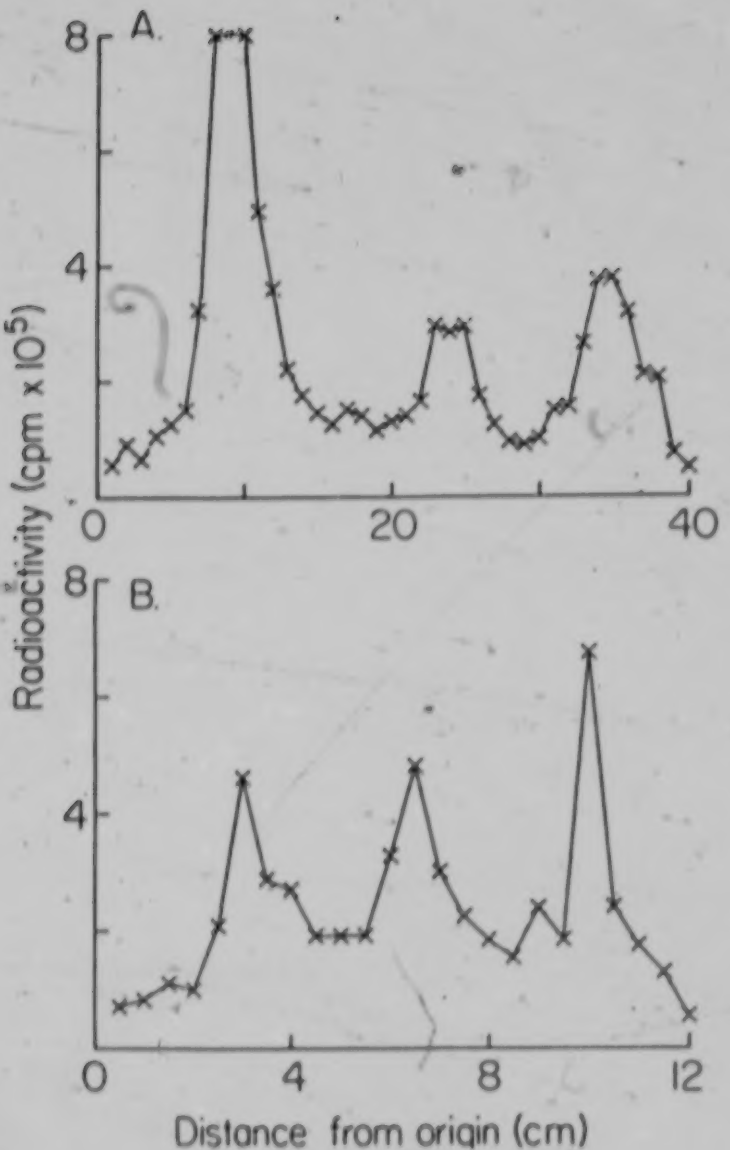


Fig. 2.3. ^{3}H-STX purification. Radioactivity profiles from high voltage electrophoresis. A. STX as purified by J.B. by paper electrophoresis. B. STX as purified by L.B. by thin layer electrophoresis. Tritiated STX was found in the third peak in both experiments, see text for details.

followed the competitive binding assay method of Reed and Raftery (1976). The specific binding - considered to be the binding displacable by 1 μM TTX - of several unknown concentrations of ^{3}H-STX was measured in the presence of 0,1,3 and 8 nM STX in a rapid filtration assay, as described below (section 2.7). From a double reciprocal plot of the resulting data, using Michaelis-Menton equations, the concentration of ^{3}H-STX can be calculated (Fig. 2.5A). In the example shown the concentration was 0.66 μM.

Radiochemical purity was calculated using an adaptation of the method of Catterall and Morrow (1978). A low concentration of ^{3}H-STX was incubated with increasing aliquots of rat brain homogenate in the absence and presence of 1 μM TTX. The reaction was terminated by centrifugation at top speed in a bench top Microfuge and aliquots of the supernatant taken for scintillation counting. Specific binding of ^{3}H-STX to the brain pellet could be determined from the difference between the supernatant cpm in the presence and absence of TTX. With concentrations of brain homogenate protein greater than 100 mg in the 1 ml incubation volume, specific ^{3}H-STX binding reached a plateau, indicating that all the STX was now bound by sites in the brain homogenate (Fig. 2.5B). Therefore, since the bioactivity of the supernatant was now zero, none of the supernatant radioactivity was associated with STX. The ratio between the cpm

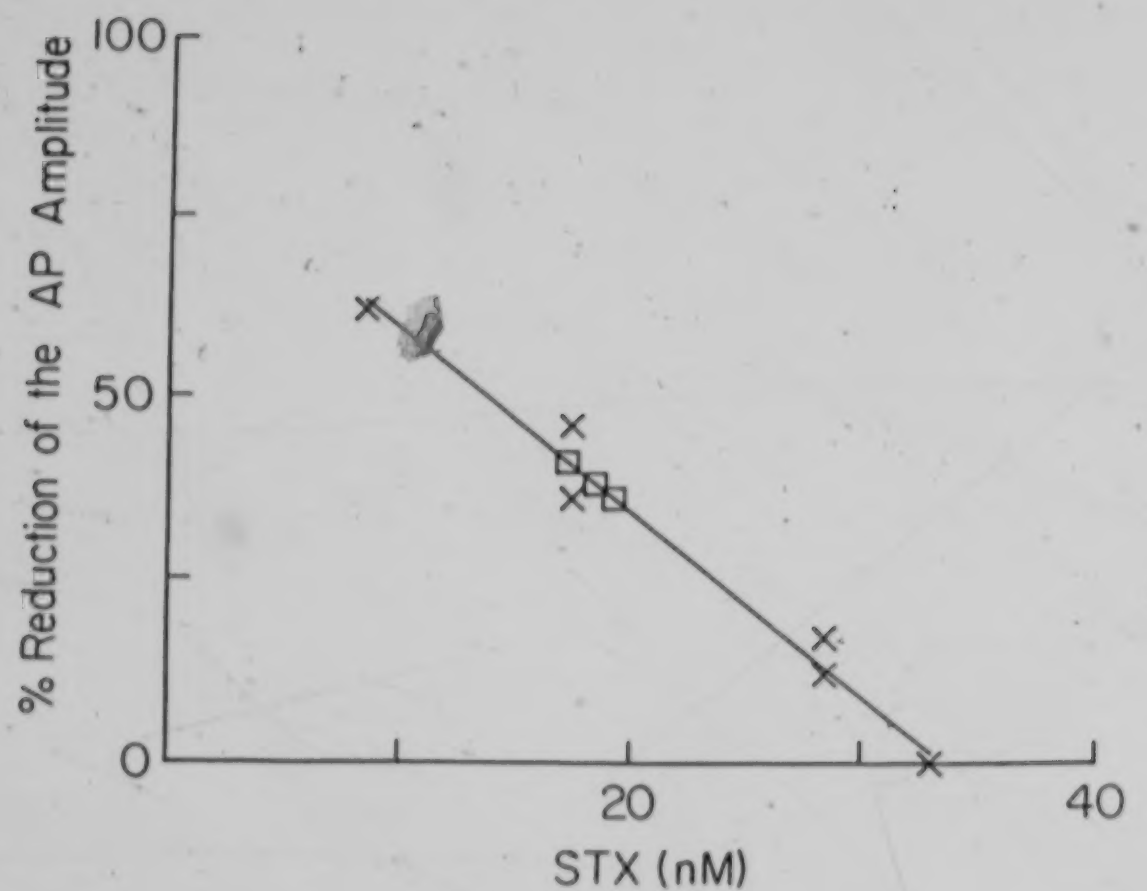


Fig. 2.4. STX dose response curve for reduction of the action potential amplitude in desheathed frog sciatic nerve. Response to known (X) concentrations of STX and to the unknown ^{3}H-STX (□).

specifically bound and the the sum of the nonspecifically bound and supernatant cpm gives the radiochemical purity. In the example shown the apparent radioactivity was 34 Ci/mmole. Fifty percent of the tritium was not specifically bound, therefore the radiochemical purity was 50% and the true radioactivity was thus 17 Ci/mmole. These values are in reasonable agreement with those obtained by Dr. Baumgold in parallel, independent assays with an aliquot of the same tritiation batch of ^{3}H-STX from Amersham.

2.3.4 Scorpion toxin: iodination and purification. ScTX (Leiurus quinquestriatus, Sigma Chemical Company) was purified (Catterall, 1976b) and iodinated (with ^{125}I) by Dr. J. Baumgold by a lactoperoxidase-catalysed iodination (Catterall, 1977). The purified toxin was lyophilized from 5 mM potassium phosphate, pH 5.5 and dissolved in 25 µl of 40 mM potassium phosphate, pH 5.5 containing 15 nmole NaI and 2 mCi (0.9 nmole) carrier-free Na^{125}I. The reaction was initiated by the addition of 5 µl of a 250 µg/ml solution of lactoperoxidase and 5 µl of 1.0 mM H_2O_2. At 15 minute intervals, two more additions of lactoperoxidase and H_2O_2 were made, so that the total amount of H_2O_2 equaled the amount of NaI (15 nmoles). The reaction was quenched with the addition of 10 mM ammonium acetate, 1 mg/ml BSA, pH 7.

The iodinated ScTX was isolated by column chromatography on a Amberlite CG-50 ion exchange column,

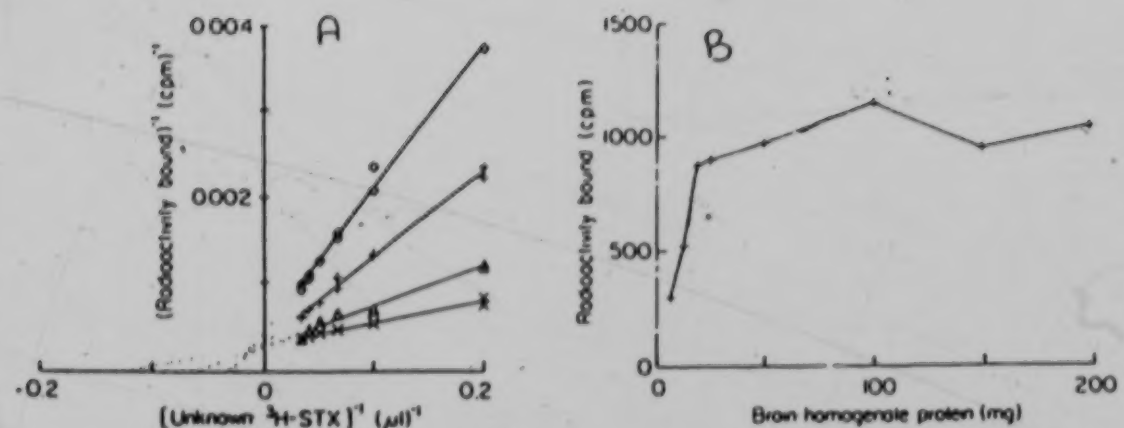


Fig. 2.5. Concentration and radiochemical purity of ^{3}H-STX. A. Double reciprocal plot of specific ^{3}H-STX binding to rat brain in the presence of 0 (X), 3(△), 5(+), 8(◇) nM STX. Maximum numbers of binding sites (Bmax) and dissociation constant (Kd) of the radiolabelled STX binding can be determined from the y and x intercepts, respectively:

y intercept = 1/Bmax

x intercept = -1/(Kd+[unlabelled STX])

and the values obtained used in the Michaelis-Menten equation:

$$y = \frac{Kd + [STX]}{Bmax} \left[\frac{1}{^3H\text{-}STX}\right] - \frac{1}{Kd+[STX]}$$

to determine the [^{3}H-STX]. B. Saturation of specific binding of ^{3}H-STX to rat brain. At concentrations of brain homogenate above 100mg, no further increase in specific binding was observed, indicating that all the ^{3}H-STX was bound by brain sites. At this concentration, 50% of the radioactivity remained unbound and, therefore, the radiochemical purity was 50%.

equilibrated in 10 mM ammonium acetate, 1 mg/ml BSA, pH 8.5. The column was washed first with 10 mM ammonium acetate to remove the unreacted I^- and then eluted with a linear gradient from 60 mM to 120 mM ammonium acetate, 1 mg/ml BSA, pH 8.5. The isolated ^{125}I-ScTX was characterized by Dr. Baumgold as described above for ^{3}H-STX but using the activation of ^{22}Na uptake by ScTX in cultured cells as a bioassay (Baumgold et al., 1983a).

2.3.5 Botulinum toxin. BoTX was the kind gift of Dr. W. Dryden, of this department, who had received it from Dr. E. Schantz. The toxicity had been calculated by Dr. Schantz as 0.033ng/ mouse LD_{50}. The toxin was diluted in water to give a stock solution of 0.357 µg/µl and stored in 50 µl aliquots in a foil wrapped container at 4°C. For injection, 1:1000 dilutions of an aliquot of this stock were made into normal saline. Stored this way, the toxin retained its potency for at least 3 years (the duration of this study). After each experiment, excess toxin was inactivated by boiling before being discarded.

2.4 Muscle homogenate and crude membrane preparations.

In all cases, animals and the relevant hindlimb muscles were weighed prior to muscle homogenization. The muscles were blotted briefly on gauze and weighed to obtain the muscle wet weight.

2.4.1 Rat muscle homogenate. Rat t.s. muscles were dissected out, trimmed of fat and connective tissue and transferred immediately to cold buffer solution on ice at

a concentration of 10 ml/g muscle. The soleus muscle was not included with the t.s. muscles because of previous reports and the results of preliminary studies in this laboratory which indicated that the time course of the effects of denervation and BoTX-treatment as well as the density of the ^{3}H-STX binding sites in these slow muscles is generally different than for fast muscles (c.f. Tonge, 1974; Hansen-Bay & Strichartz,1980, Bambrick et al., 1983). The muscles were minced roughly and then 10-20 ml aliquots were homogenized in two 30 second bursts of a Polytron homogenizer. The homogenate was filtered through 4 layers of surgical gauze to remove connective tissue. The filtrate, which is the muscle homogenate, was either frozen at -70 °C or used immediately in ^{3}H-STX and ^{125}I-BTX binding assays.

For the assay of ^{3}H-STX binding sites, muscles were prepared in a Na-free solution containing 130 mM choline chloride, 5.4 mM KCl_2, 5.5 mM glucose, 0.89 mM $MgSO_4$, 1.8 mM $CaCl_2$ and 50 mM Hepes adjusted to pH 7.4 with Tris base (Buffer A) (Sherman & Catterall, 1982). Specific ^{125}I-BTX binding in this buffer was very low, possibly due to the presence of choline, which is a weak partial agonist of nicotinic ACh receptors (Lawrence & Catterall, 1981). Consequently, muscle for ^{125}I-BTX binding was prepared in a different buffer containing 50 mM sodium phosphate at pH 7.4 to which protease inhibitors, 1 mM 1,10 phenanthroline, 1 uM pepstatin A, 0.1 mM

phenylmethylsulfonyl fluoride and 1 mM iodoacetamide were added (Buffer B) (Festoff & Engel, 1974; Bambrick & Gordon, 1985b). Although protease inhibitors do not appear to be required for preservation of ^{3}H-STX binding activity in muscle homogenates (Sherman & Catterall, 1982), preliminary experiments in this laboratory showed improved ^{125}I-BTX binding when protease inhibitors were included.

2.4.2 Rat muscle membrane preparation. The homogenate was further purified in some experiments by adapting procedures of Colquhoun et al. (1974) and Barchi, Weigle, Chalikian & Murphy (1979) to yield a crude membrane fraction. Following the homogenization, an initial 1000xg centrifugation removed connective tissue, tissue chunks and whole red blood cells. The supernatant was centrifuged twice at 100,000xg to yield a crude membrane pellet which was resuspended in buffer and, again, either frozen at -70 C or used immediately in ^{3}H-STX binding assays.

2.4.3 Cat muscle homogenate preparations. Cat muscle homogenates were prepared by Dr. L. Eldridge at UCLA. Lateral and medial gastrocnemius, soleus and plantaris muscles were removed from cats under pentobarbitol anaesthesia. The muscles were homogenized as described in Section 2.2.2 for rat muscles. Prior to filtration through gauze the homogenates were frozen and stored at -70 ^{o}C until they were transported to the University of Alberta on dry ice. These homogenates were then again stored at -70 ^{o}C until needed. The samples were thawed and filtered

just prior to the radioligand binding assays and only used if the samples were all still frozen on arrival in Edmonton (only one shipment of 3 arrived with any samples thawed and all samples from that shipment were discarded). To control for the possibility that preparation at UCLA and the subsequent shipping might alter the binding of radioligands to the muscles, the maximum binding (Bmax) of ^{125}I-BTX to 22 day and 8 month denervated gastrocnemius and plantaris muscle homogenates from UCLA was compared to that of 2 month denervated muscle homogenates prepared at this university. Since the maximal binding of ^{125}I-BTX to the fresh or frozen and shipped muscles was comparable, it was clear that the shipping did not introduce any significant artifacts.

2.5 Rat brain preparations.

2.5.1 Brain homogenate preparations. For studies of the development of ^{3}H-STX binding sites in rat brain, freshly excised brains were homogenized in ice cold Buffer B using a Teflon-glass homogenizer. The homogenate was centrifuged twice at 100,000xg for 45 minutes. The final pellet was resuspended in buffer and used for ^{3}H-STX binding (Hartshorne & Catterall, 1981; Bambrick, Baumgold & Gordon, 1983; Baumgold et al., 1983).

Rat brain homogenates for use in determinations of ^{3}H-STX concentration and purity (c.f. section 2.3.3) were homogenized in 0.25M sucrose. The homogenate was centrifuged at 40,000xg for 15 min. The pellet was

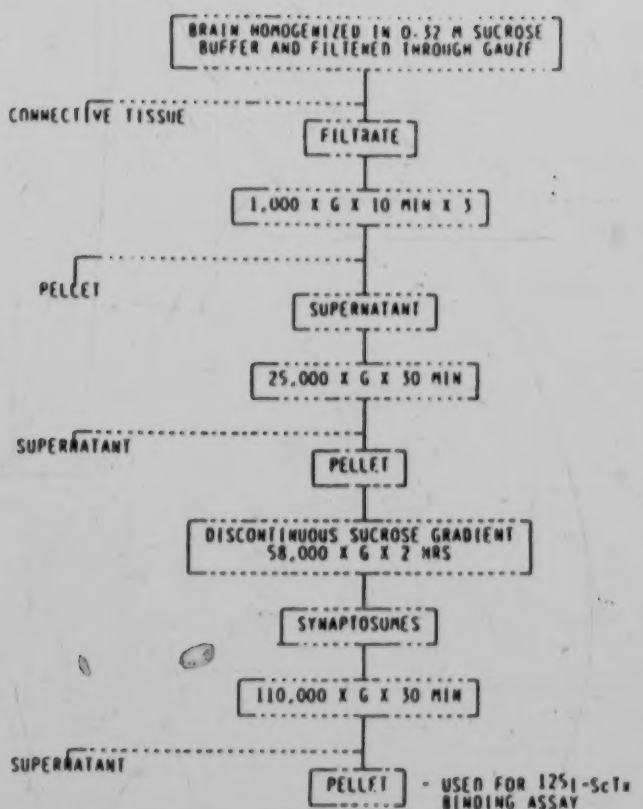


Fig. 2.6. Brain synaptosomal preparation for ^{125}I-ScTX binding. Synaptosomes isolated from the discontinuous sucrose gradient were concentrated by centrifugation and used for the binding assays.

resuspended in Buffer A minus the $CaCl_2$.

2.5.2 Rat brain synaptosome preparation. Due to the voltage dependence of ScTX action (Catterall, 1977) binding of this toxin could only be measured using a synaptosomal preparation. Freshly excised rat brains were processed as shown in Fig. 2.6 (Catterall, 1977). The final fraction was used for ^{125}I-ScTX binding (Bambrick et al., 1983).

2.6 ^{125}I-BTX binding protocol.

Muscle homogenates in Buffer B were incubated with ^{125}I-BTX in the absence or presence of 1 μM unlabelled BTX for periods of up to 30 minutes. The reaction was terminated by rapid filtration over a vacuum and the filters washed, air-dried and counted in a Beckman Gamma 8000 counter. Aliquots of the incubate were taken for determinations of the free ^{125}I-BTX concentration.

2.6.1 Choice of filter. Initially, glass fibre filters (Whatman, GF/B), commonly employed in homogenate filtration assays, were used. However, there was very high specific binding to the GF/B filters in the absence of any muscle protein. This non-biological specific binding declined, instead of increasing, with increasing muscle concentration in the incubate and could not be eliminated by the presence of BSA (Sigma) in the incubate nor by pre-soaking the filters in BTX. When the specific and nonspecific binding of ^{125}I-BTX to different filter types in the absence of muscle protein was compared,

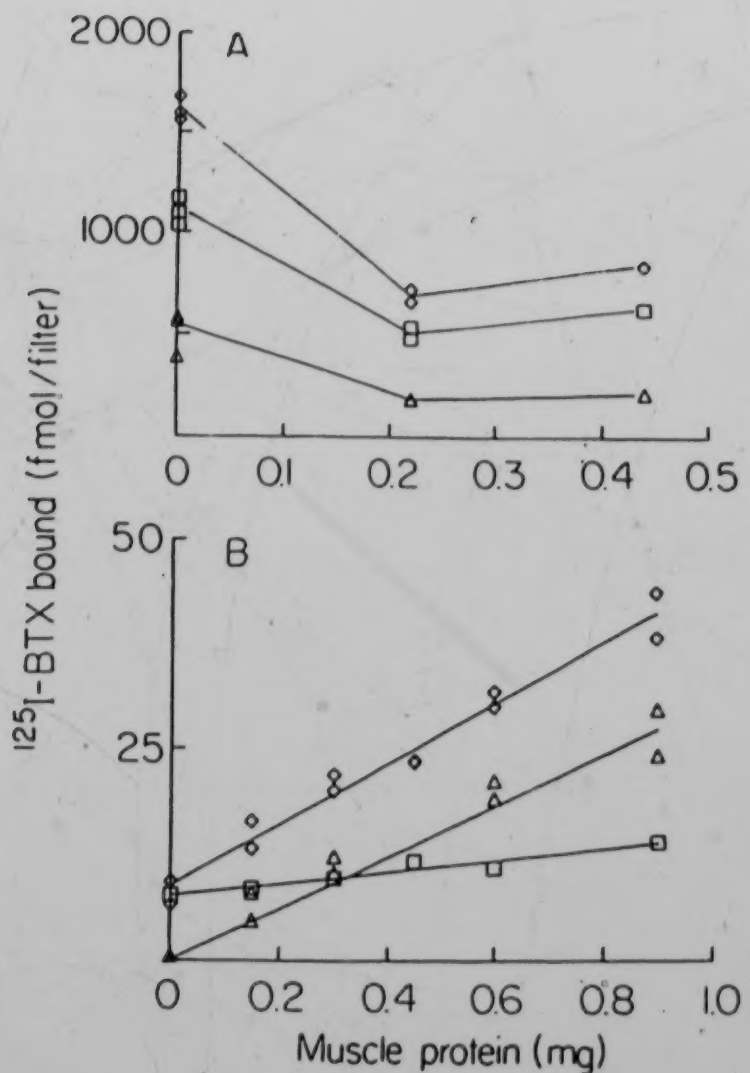


Fig. 2.7. ^{125}I-BTX binding with increasing denervated muscle protein for GF/B (A) or DE-81(B) filters. Total (◇) and (□) nonspecific ^{125}I-BTX binding in the absence or presence of 4 um BTX. Specific binding (△) is taken as the difference between total and nonspecific binding. Note the apparent specific binding to GF/B filters in the absence of muscle protein. In contrast, there was no "specific" binding to DE-81 filters alone where specific binding increased linearly with increasing muscle protein.

diethylamino cellulose (Whatman DE-81) filters showed no non-biological specific binding and the nonspecific binding of ^{125}I-BTX was low (Fig.2.7A,B). Since GF/B filters carry a slight negative and DE-81 filters a slight positive charge, it is possible that the difference in filter performance is related to differences in their ionic interactions with the toxin (Bambrick & Gordon, 1986a). The addition of BSA to the incubation medium and of both BSA and 0.1 μM BTX to the buffer in which the filters were soaked reduced the nonspecific binding and these conditions were therefore employed throughout.

2.6.2 Muscle protein and ligand concentrations.

To establish the optimal conditions for ^{125}I-BTX binding assays, increasing amounts of muscle homogenate in Buffer B were incubated with ^{125}I-BTX in the absence or presence of unlabelled BTX. The total (without BTX), nonspecific (with BTX) and specific (the difference between the two) ^{125}I-BTX binding was linear for a range of homogenate protein up to 2 mg and reached a maximum before 15 minutes of incubation.

Unlike STX, BTX binds almost irreversibly so that the equilibrium situation represents complete receptor occupancy (Colquhoun et al., 1974). Therefore ^{125}I-BTX binding increases with increasing ^{125}I-BTX concentration until the receptors are saturated at concentrations of ^{125}I-BTX above 20 nM. To ensure that sufficient BTX was used to displace the maximum ^{125}I-BTX, aliquots of muscle

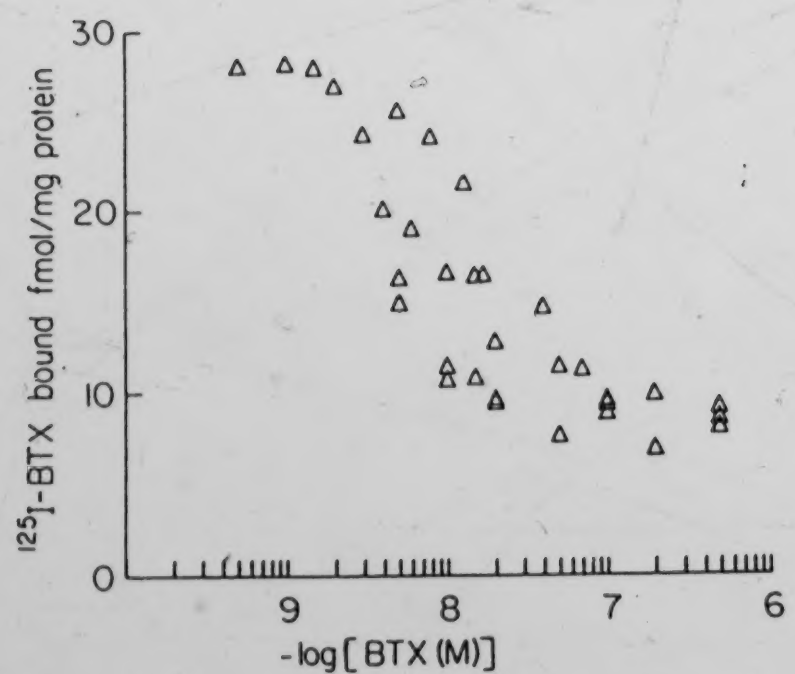


Fig. 2.8. ^{125}I-BTX binding to a muscle homogenate prepared from 14 day denervated rat hindlimb muscles plotted as a function of increasing concentration of unlabelled BTX. The specific ^{125}I-BTX binding is the maximal ^{125}I-BTX binding protected by concentrations of BTX in excess of 0.1 μM.

homogenate were incubated with 30 nM ^{125}I-BTX and increasing concentrations of BTX until the total binding plateaued at a low and steady level at [BTX]>1μM (Fig.2.8).

2.6.3 Assay methodology. ^{125}I-BTX binding was assayed by incubating 0.5-1.5 mg muscle protein with 30 nM ^{125}I-BTX in Buffer B containing 1 mg/ml with BSA in the absence or presence of 4 μM BTX. Aliquots of the incubation medium were taken for determination of the total radioligand concentration. The free radioligand concentration was calculated by subtracting the amount bound from the total amount of radioactivity in the incubation medium. After 30 minutes, the incubation was terminated by rapid filtration through DE-81 filters pre-soaked in Buffer B, 1 mg/ml BSA, 0.1 μM BTX. The filters were washed with five 3 ml volumes of ice cold 50 mM sodium phosphate, pH 7.4 to reduce the nonspecific binding. Because the binding of BTX to the ACh receptor is nearly irreversible, the repeated washing does not lead to a reduction in the specific ^{125}I-BTX binding. The filters were air dried and counted in a Beckman Gamma 8000 counter.

The specific binding was obtained by subtracting the ^{125}I-BTX bound in the presence of 4 μM BTX from the ^{125}I-BTX bound in the absence of BTX. As shown in Table 2.1, specific binding was routinely assayed by incubating 3-4 aliquots of muscle homogenate with 30 nM ^{125}I-BTX in the absence and 3-4 aliquots in the presence of 4 μM BTX.

^{125}I-BTX bound (fmol/mg protein)

Days of	a) Replicate Assays for 1 muscle			b) Assays from 2-6 Muscles		
	Total	Nonspecific	Specific	Total	Nonspecific	Specific
Denervation						
4	32.7±4(3)	25.1±2(3)	7.6±2(3)	27.2±2(4)	19.8±2(4)	7.4±1(4)
7	51.2±2(3)	25.0±3(3)	25.8±4(3)	45.9±3(4)	24.0±2(4)	22.0±2(4)
21	60.8±1.2(4)	21.5±1.2(4)	39.6±2(4)	62.0±1(6)	23.0±1(6)	39.0±3(6)

TABLE .1 Mean ± SE (n) of ^{125}I-BTX bound (fmol/mg protein) for denervated triceps surae muscles incubated with 30 nM ^{125}I-BTX in the absence (total binding) and presence (nonspecific binding) of 4 μM BTX for a) an example of 3-4 replicate assays for one muscle and b) 2-6 muscles. In b), the means obtained from the replicate assays for each muscle have been summed to obtain the mean for all muscles. Note that the difference (Specific binding) is the same in a) and b)

Nonspecific binding was statistically different ($p<0.01$, t-test) from total binding so that specific ^{125}I-BTX binding could be readily calculated as the difference.

2.7 ^{3}H-STX binding protocol

2.7.1 ^{3}H-STX binding to muscle. The protocol for ^{3}H-STX binding follows the procedure of Sherman & Catterall (1982) adapted by Bambrick and Gordon (1985a). Aliquots of muscle homogenate or crude muscle membrane fraction were incubated with ^{3}H-STX in the absence or presence of an excess (1 μM) TTX in Buffer A. Specific ^{3}H-STX binding to muscle homogenate increased linearly over a range of 0-2.0 mg. The amount of protein used in an assay was always chosen to fall within this range. There was no specific binding to the GF/B filters used in the absence of protein.

Maximum binding was reached after 15 minutes so homogenates were routinely incubated for 25-30 minutes. The reaction was then terminated by rapid filtration through GF/B filters. The maximum loading capacity of the filter was determined anew for each preparation as being the greatest amount of homogenate in solution that would, in a 3 ml volume, be filtered over vacuum in 2 seconds. The concentration of homogenate used was always below this value.

The filters were washed in less than 10 seconds with three 4 ml volumes of ice cold buffer, 163 mM choline chloride, 5 mM Hepes, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, pH 7.4.

This reduced the nonspecific binding without affecting specific ^{3}H-STX binding. An aliquot of the incubation medium was counted and the free ^{3}H-STX concentration was determined as described for ^{125}I-BTX binding. The filters were air dried and transferred to glass scintillation vials containing 10 ml of Aquasol (New England Nuclear) or ACS (Amersham). After 3-4 hours in the dark, the samples were counted using either a Beckman LS 7500 or a Beckman LS 6800 liquid scintillation counter. Quench curves were calculated using known amounts of ^{3}H in these volumes of scintillant with varying amounts of H_2O. Percent counting efficiency was plotted against H#. This relationship was used to convert cpm per filter or per aliquot into dpm. The H# is calculated by the scintillation counter as the shift in the pulse distribution of the Compton electrons, produced in the presence of an internal gamma source, by quenching in the sample.

Total ^{3}H-STX binding (without TTX) and nonspecific ^{3}H-STX binding (with TTX) were plotted as a function of the free ^{3}H-STX concentration. Specific binding was obtained by subtracting the non-specific component from the total binding and plotted as a function of the free ^{3}STX concentration. This function was fitted, using least mean square criteria, with a hyperbolic curve of the form $Y=(P_1X)/(P_2+X)$ where P_1 is the maximum number of binding sites (Bmax) and P_2 is the dissociation constant (Kd). The Bmax and the Kd were also calculated by replotting the

data as a Scatchard plot where the slope is -1/Kd and the x-intercept is the Bmax. Only values from experiments in which the correlation coefficient for the linear regression line of the Scatchard plot was greater than 0.7 were accepted. Estimates of Bmax and Kd by either method were always in good agreement.

2.7.2 **^{3}H-STX binding to rat brain.** ^{3}H-STX binding to rat brain was carried out under essentially the same conditions as described above for muscle with the following exceptions. Buffer B was used in all the studies of the development of ^{3}H-STX binding sites in neonatal and adult rat brain (Baumgold et al., 1983; Lombet et al.,1983). The brain protein concentration was always kept between 0 and 1.0 mg/ml protein, all filters were washed 3 times with 50mM phosphate buffer, pH 7.4 (Fig. 2.9A,B). In the experiments for the determination of ^{3}H-STX concentration and purity, calcium-free Buffer A was used in the filtration assays and the filters were washed 3 times with ice-cold 163 mM choline chloride, 5 mM Hepes, 0.8 $MgSO_4$, 1.8 $CaCl_2$, pH 7.4 (Catterall & Morrow, 1978).

2.8 ^{125}I-ScTX binding to rat brain synaptosomes.

The parameters for ^{125}I-ScTX binding including synaptosome concentration, incubation time and wash volume were optimized as described for ^{3}H-STX and ^{125}I-BTX and following the method of Catterall(1977) (Fig.2.9 .C,D). Aliquots of the supernatant were incubated with increasing concentrations of ^{125}I-ScTX in the absence or presence of

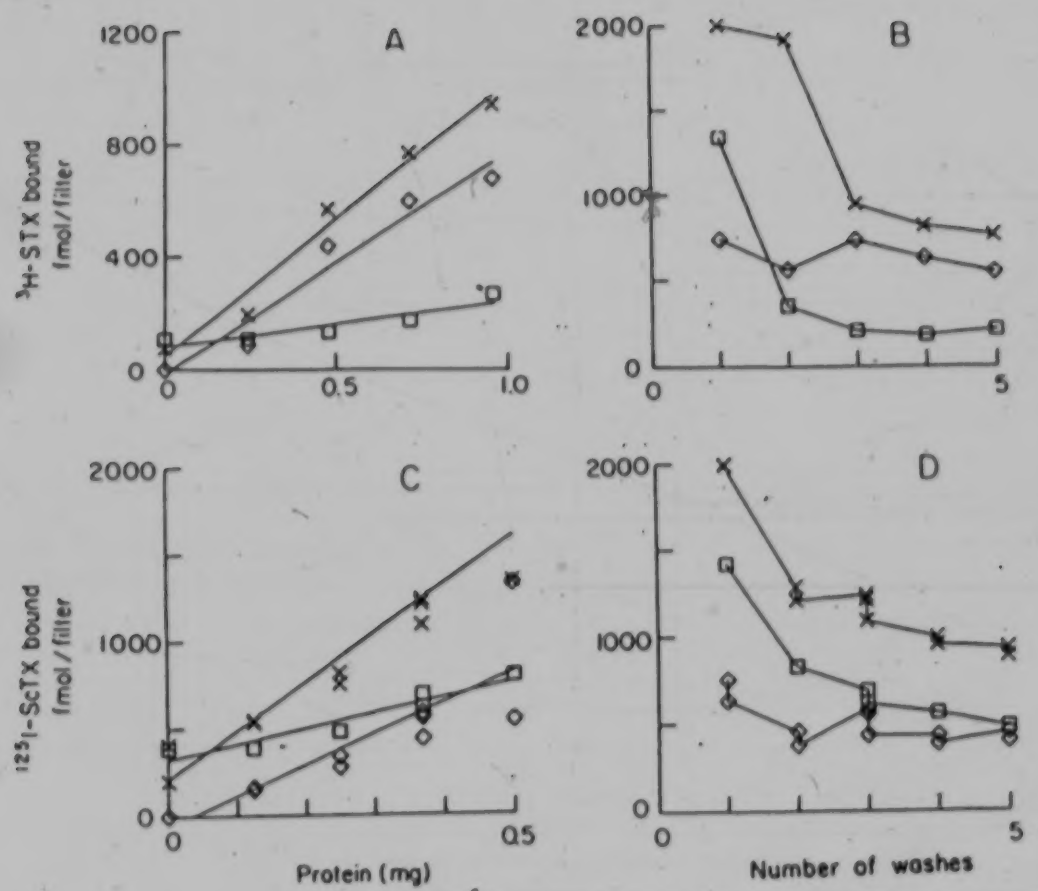


Fig. 2.9. ^{3}H-STX (A,B) and ^{125}I-ScTX (C,D) binding to adult rat brain homogenates and synaptosomes, respectively. A,C total (X), nonspecific (□) and specific (◇) binding increase linearly with brain protein. B,D Increasing numbers of filter washes reduced nonspecific but not specific binding over 1-5 washes.

100 nM unlabelled ScTX for 5-30 minutes. The final incubation volume was adjusted to 0.5 ml with Buffer A made without $CaCl_2$ and containing 1 mg/ml BSA. Aliquots of the incubate were taken for counting and at the end of the incubation period the solution was filtered through Reeve-Angel glass fibre filters (934-AH) which had been pre-soaked in wash buffer (163 mM choline chloride, 5mM Hepes, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 1 mg/ml BSA). The filters were washed with three 2.5 ml volumes of wash buffer, air dried and counted. Total filtration time was always less than 10 seconds (Bambrick et al.,1983).

2.9 Protein determination.

Protein concentration was determined for muscle and brain preparations following the method of Lowry, Rosebrough, Farr & Randall (1951) with BSA (Sigma) as standard. Over the range of protein concentrations used (0-0.5 mg/ml), the standard curve only became noticeably curvilinear above 0.4 mg BSA/ml. Concentrations of brain or muscle protein were chosen to fall within the linear range of the standard curve and showed a linear increase in absorbance with increasing protein concentration (Fig. 2.10). Although different purified proteins do have different sensitivities to the Lowry reaction (Maddy, Dunn & Kelly, 1972), the difference in chromogenicity is much less marked for mixtures of proteins such as those which are found in homogenates of various tissues (Lowry et al. 1951).

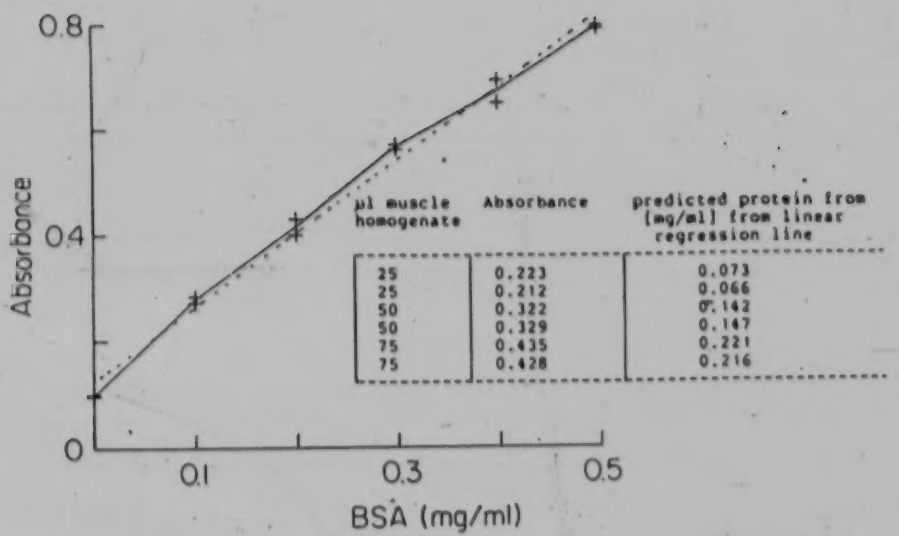


Fig. 2.10. Linear relation between Absorbance and either BSA concentration (compare solid line connecting data point means and dotted line of calculated linear regression line, RO=0.99) or muscle homogenate concentration (inset).

2.10 Histology

Muscle fibre diameters were determined from analysis of photomicrographs of muscles stained for alkali ATPase activity, which gave good resolution of the fibre perimeters.

2.10.1 Muscle frozen sections. Rat t.s. muscles from adult and neonatal animals were carefully excised, cut into 0.5-1.0 cm lengths and mounted, two muscles per cork, on corks using the mounting medium OCT (Tissue Tek, Miles Laboratories Inc). The muscles were flash frozen by being plunged into isopentane cooled by liquid nitrogen to -160 °C. The frozen blocks were transferred immediately to storage at -70 °C (Dubowitz & Brooke, 1973).

For the preparation of sections, the blocks were transferred to a cryostat and warmed to -20°C. 8-10 μm sections were cut by Ms Serap Erdibil. Muscle cross-sections were cut and the sections were picked up on glass coverslips and air dried for an hour before being stained.

2.10.2 Alkali ATPase stain. The alkali ATPase stain was adapted from the method of Guth & Samaha (1970). The muscle sections were fixed in modified 2% formalin for 5 minutes followed by a 1 minute wash in 18 mM $CaCl_2$, 0.1 M Tris buffer, pH 7.8. The sections were kept in the alkali pre-incubation medium, 18mM $CaCl_2$, 0.1M glycine-NaOH, pH 10.4, for 20 minutes and rinsed with 2X30-second washes in distilled water, pH 8.5-9.0 (with KOH). Separate sections were then reacted with 2.7 mM ATP, 50 mM KCl, 18 mM $CaCL_2$

in glycine-NaOH, pH 9.4 for 20, 30 or 60 minutes at 37°C. The reaction was terminated with 3X30-second washes of cold 1 % $CaCl_2$. The reaction product was visualized by incubating the sections for 3 minutes in 2% $CoCl_2$, followed by 4X30-second washes in distilled water, pH 8.5-9.0, a 3 minute incubation in 1% $(NH_4)_2S$ and a final 3X30-second wash in pH 8.5-9.0 distilled water. All sections were air dried and mounted on glass slides with glycerin jelly.

2.10.3 Photography and quantification of histochemical data. The stained slides were photographed through a Leitz 4mmPS microscope. Photomicrographs of a final magnification X315 to X760 were used such that there were approximately 100-200 muscle fibres per 8" by 10" print.

Muscle fibre diameters were measured using a Microplan 2 Image Analysis System (Laboratory Computer Systems Inc., Cambridge, MA) with the kind permission of Dr. K. Bagnell and the Department of Anatomy, University of Alberta. Using this method, a cursor was moved around the circumference of the fibre and a value for the fibre cross-sectional area was calculated by the Microplan. From this area, a value for the fibre radius was calculated considering that area= πr^2. Another value for the fibre radius can be obtained by moving the cursor across the muscle fibre's least full diameter. These values were stored on the Microplan and the mean, standard deviation of the mean, and number of fibres were calculated.

It was difficult to decide which value for the diameter was more appropriately used for normalization of binding site data. Due to the likelihood that some of the sections were oblique relative to the long axis of the muscle fibre, the diameter calculated from the actual photomicrograph is almost certainly an over-estimation. If the muscle fibres were perfectly cylindrical, the least full diameter would always be correct. However, examination of the photomicrographs revealed that the muscle fibres were genuinely irregular in their shape and, therefore, that the least full diameter would underestimate the true fibre radius. Upon computation of both values it was found that the radius calculated from the least full diameter was always 0.7-0.8 of the radius calculated from the cross-sectional circumference of fibres from muscles from control, nerve section or BoTX-treated rats 1-8 weeks of age. Since there was no apparent change in this ratio with age or treatment, normalization of binding data by either value for the radius would lead to qualitatively the same results. The latter value, the radius computed from the circumference was preferred because manual tracing around a fibre circumference does not require the subjective choice on the part of the operator which selection of the least full diameter does.

2.11 Data analysis

Binding site data were normalized per mg wet weight brain or muscle, or per mg preparation protein.

Comparative binding site densities were calculated from the muscle fibre diameter, and the surface area:volume ratio of a cylinder, which is 2πx radius x length:πradius^2 x length or 2:radius.

All comparisons between control, denervated and BoTX-treated groups were made using an analysis of variance. Comparisons between denervated and BoTX groups were made using both an analysis of variance and the Student's t-test. Mean Bmax and Kd values obtained for triplicate measurements of toxin binding to muscle or brain preparations from individual experiments were treated as discrete data points in terms of calculating mean values for several experiments.

All regression lines were calculated according to the method of least mean squares (Edwards, 1976). The significance of the fit of the regression lines to the data were tested by using the correlation coefficient in the t-test and by calculating the significance of the difference of the slope from zero. A non-linear regression analysis program was employed for computation of hyperbolic and exponential curves. This program is available in the B MDP package of programmes for biomedical applications developed at the Health Sciences Faculty of the University of California, Los Angeles. The validity of describing the data with an exponential curve was tested in each instance by calculating the significance of the correlation coefficient for

appropriate logarithmic transformation.

CHAPTER THREE: ACETYLCHOLINE RECEPTORS AND SODIUM CHANNELS IN DENERVATED AND IN BOTULINUM TREATED ADULT RAT MUSCLE.

3.1 Introduction.

Denervation of adult skeletal muscle results in the appearance of wide-spread extrajunctional ACh sensitivity (Kuffler, 1943; Miledi, 1960; Pumplin & Fambrough, 1982) and of action potentials which are partially resistent to TTX, a neurotoxin which normally blocks Na channels (Redfern & Thesleff, 1972a,b; Pappone, 1980). The change in muscle chemosensitivity is due to the incorporation of newly synthesized ACh receptors into the extrajunctional membrane, measured as a dramatic rise in the number of ^{125}I-BTX binding sites (Colquhuon et al., 1974; Askmark et al., 1982). The molecular basis for the TTX-resistant action potentials now seems to be the appearance of Na channels with very low affinity for TTX (Rogart & Regan, 1985). Whether the appearance of these low affinity sites for toxin is accompanied by a decline in numbers of high affinity sites has not been established.

The mechanism of the neuronal control of these membrane proteins underlying muscle chemosensitivity and excitability is also unclear. Are they regulated by neuromuscular activity or by "an influence of the nerve not mediated by impulses" (Miledi, 1963) (see Chapter 1 for a more detailed review)? Presynaptic blockade of ACh release with BoTX, increases ACh sensitivity (Tonge, 1974; Pestronk et al., 1976; Mathers & Thesleff, 1978; Brown et

al., 1982) and induces the appearance of action potentials partially resistant to TTX (Mathers & Thesleff, 1978). This implies a role for neuromuscular activity in the mediation of the neuronal regulation, but whether it is the only factor is unclear. The lesser effects of BoTX as compared to denervation in several studies have been interpreted as evidence for a non-activity mediated component of the neurotrophic control of muscle.

In this study, the role of neuromuscular activity in the regulation of muscle ACh receptors and Na channels was examined by a detailed quantification and determination of the time course of the changes in ^{125}I-BTX and ^{3}H-STX binding in denervated and BoTX-treated muscles.

3.2 Results

3.2.1 Muscle atrophy. Atrophy is historically one of the earliest reported changes consequent to denervation of skeletal muscle. Fig. 3.1 shows that after sciatic nerve section, t.s. weight declines with time to a value of 400 mg by >3 weeks. Normally innervated muscles also atrophy after BoTX treatment to the same extent as muscles denervated for equivalent periods of time (Fig. 3.1). Muscle protein also decrease by equivalent amounts in denervated and BoTX poisoned muscles (Fig.3.2). Since adult rat weight is not significantly affected by denervation or BoTX treatment, in contrast to neonatal rat body weight (Chapters 5;8), the decline in t.s. weight normalized by rat weight decreases similarily to muscle

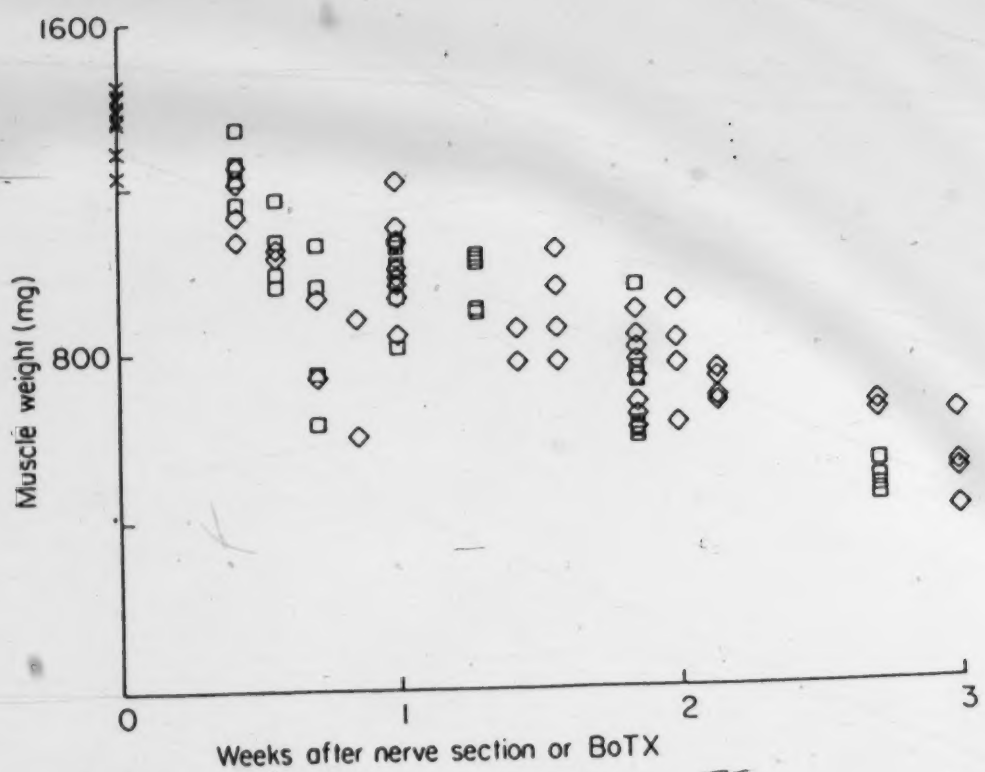


Fig. 3.1. Decrease in t.s. muscle weight from control values (X) with time after denervation (◇) or BoTX (□) injection.

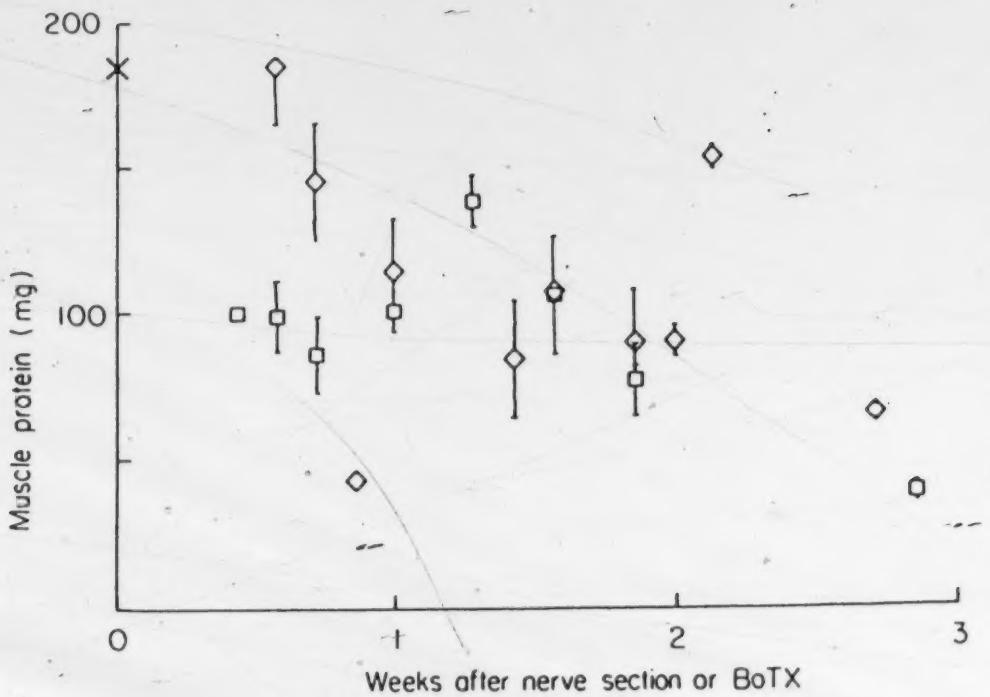


Fig. 3.2. Decrease in t.s. muscle protein content from control (X) values as a function of time after denervation (◇) or BoTX injection (□). Mean±SE, n=2-6 muscles.

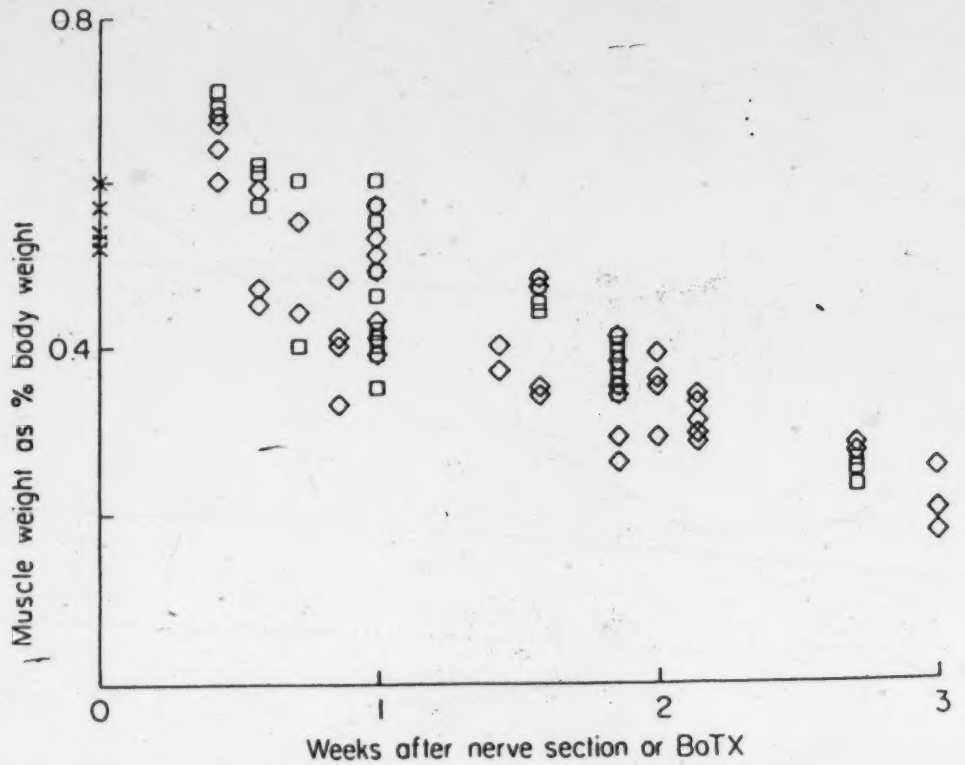


Fig. 3.3. Decrease in t.s. muscle weight as a percentage of rat body weight from control values (X)as a function of time after denervation (◇) or BoTX injection (□).

weight alone (Fig. 3.3). Muscle fibre diameter also decreased with time in denervated and BoTX treated muscles, falling to 48±0.7 μm (180) and 43±0.9 μm (191), respectively, from control values of 55±0.7 μm (200) (X±SE (180-200 fibres in 2 muscles)) by one week after treatment.

3.2.2 Effect of denervation and BoTX poisoning on number of ^{125}I-BTX binding sites. Normally ACh receptors are confined to the endplate region of a focally innervated muscle and specific binding of ^{125}I-BTX to muscle homogenate is not measurable. As is shown in Table 2.1, denervation of the muscle results in a dramatic increase in ^{125}I-BTX which, by 3-4 days after nerve section, is readily discernable above the nonspecific binding. Nonspecific binding was statistically different ($p<0.01$) from total binding so that specific ^{125}I-BTX binding could readily be calculated as the difference. The number of specific binding sites increased rapidly over the first week of denervation and had increased by an order of magnitude to attain a mean level of 39±3 fmol/mg homogenate protein, or 3.2±0.5 fmol/mg wet weight, (mean±SE, n=6) by 3 weeks (Fig. 3.4). The increase in ^{125}I-BTX binding sites was well fitted by a single exponential with an asymptotic value of 41.3 fmol ^{125}I-BTX per mg protein and a time constant of 6 days using the curve fitting program, as described in Chapter 2. A logarithmic transformation of the data which was fitted with the expontential in Fig. 3.4 ($\log(P_1-Y)$ versus time)

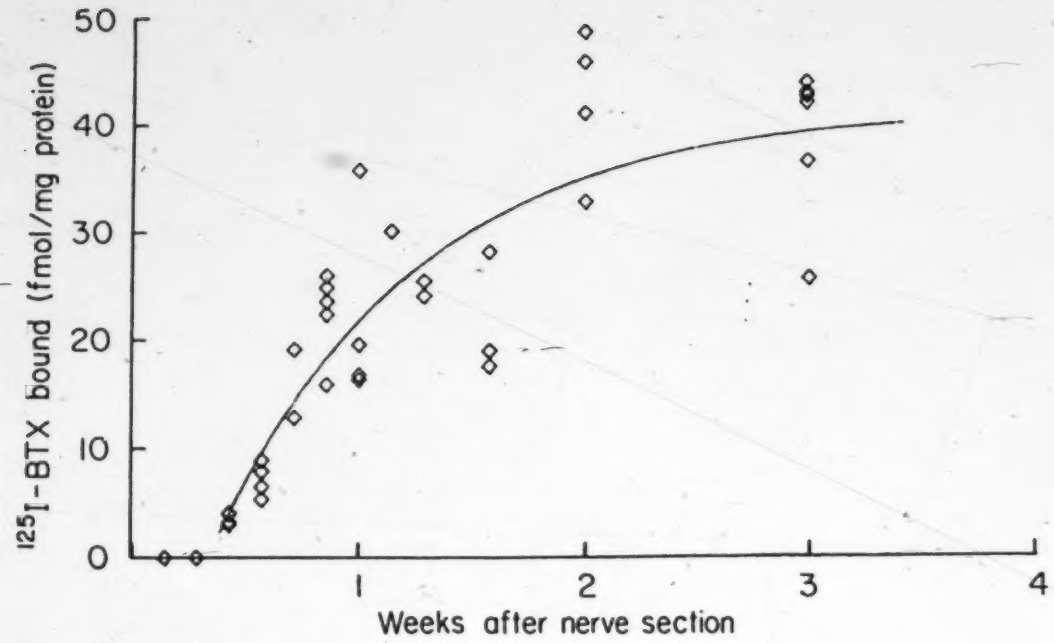


Fig. 3.4. ^{125}I-BTX binding in homogenates of adult rat t.s. muscles as a function of time after sciatic nerve section. Measurable specific ^{125}I-BTX binding was first seen 3 days after nerve section. The rise in the number of binding sites has been fitted with a single exponential of the form:

$$Y=P_1-P_2e^{-P3x}$$ (cf. Chapter 2).

The maximal asymptotic value was 41.3 fmol/mg protein and the time constant was 6 days. Each point shown is the mean of 3-4 replicate determination of binding to muscle homogenate prepared from 1 rat t.s. muscle.

can be well fitted with a straight line on a semi-logarthmic scale with a correlation coefficient of 0.82 which is significant at $p<0.01$.

The ten-fold increase in numbers of ACh receptors within the first week of denervation compares well with previous reports in denervated rat diaphragm and hindlimb muscle (Colquhuon et al., 1974; Berg & Hall, 1975; Askmark et al., 1982). The exponential rise in numbers of ^{125}I-BTX binding sites implies that ACh receptor synthesis reaches a stable level within three weeks of nerve section. As muscle protein content only declines by 25% during the first week, it is clear that this increase in site numbers requires a greatly increased synthesis of ACh receptors.

Local injection of BoTX into the hindlimb induced the appearance of measurable specific ^{125}I-BTX binding after 3 days. Numbers of ^{125}I-BTX sites continued to rise over the following week (Fig. 3.5). Complete neuromuscular blockade was usually not maintained beyond 11 days after the injection (see also Chapter 8). Two weeks after BoTX injection, the numbers of ^{125}I-BTX binding sites fell short of the levels reached following an equivalent period of denervation, concurrent with some recovery of neuromuscular transmission. Interestingly, the numbers of ^{125}I-BTX binding sites were still elevated 3 weeks after BoTX injection, by which time sufficient neuromuscular transmission had returned to elicit detectable force in response to nerve stimulation. This indicated that the

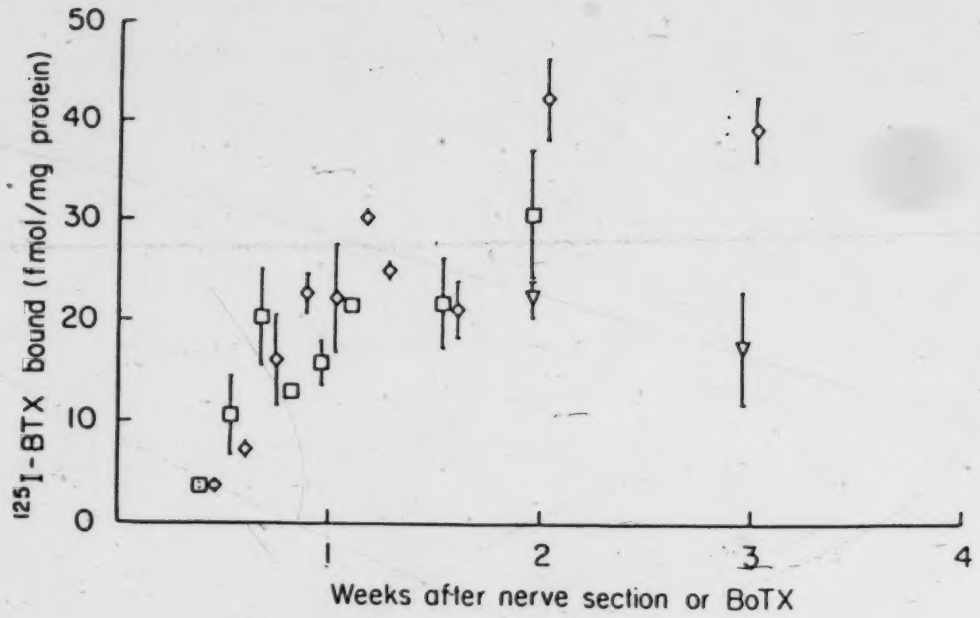


Fig. 3.5. The mean ±SE of ^{125}I-BTX binding to muscle homogenate prepared from 4-7 denervated (◇) and BoTX paralyzed (□) rat t.s. muscle as a function of time after treatment. Both treatments induced the appearance of measurable specific binding within 3 days and showed an equivalent effect in increasing the binding over the first 11 days. Note that after 14 days, in muscle treated with BoTX which had begun to recover from the neuromuscular blockade (▽), the numbers of ^{125}I-BTX sites fell short of those in muscles denervated for the same period of time.

decline in the number of ACh receptors lags behind the physiological recovery of neuromuscular transmission. This decline does, however, preceed any recovery of muscle weight - weight is still depressed 3 weeks after treatment (Fig. 3.1). Comparison of ^{125}I-BTX binding at any day of complete BoTX blockade or denervation showed that the effects of BoTX blockade and denervation were not statistically different ($p>0.05$).

The time courses of the increase in numbers of ^{125}I-BTX binding sites in denervated and BoTX paralyzed muscle were compared by plotting the increase in ^{125}I-BTX binding in Fig 3.6 as a function of time after nerve section or after BoTX injection. Over the first 11 days, when BoTX blockade is complete, the time course of the increase in ^{125}I-BTX sites after neuromuscular blockade has been compared to the time course of the increase following denervation by calculating the deviations of the data points from the exponential curve fitted to the data in Fig. 3.4. An analysis of variance showed that the numbers of ^{125}I-BTX binding sites in BoTX treated muscles did not deviate significantly more from this curve than did the numbers of sites in denervated muscles over the same time period ($p>0.05$). The rise in ^{125}I-BTX binding in the 3-11 days following BoTX blockade was, therefore, well fitted by the same exponential time course calculated for the rise in ^{125}I-BTX binding consequent to denervation. It is thus apparent that, when BoTX is effective in producing

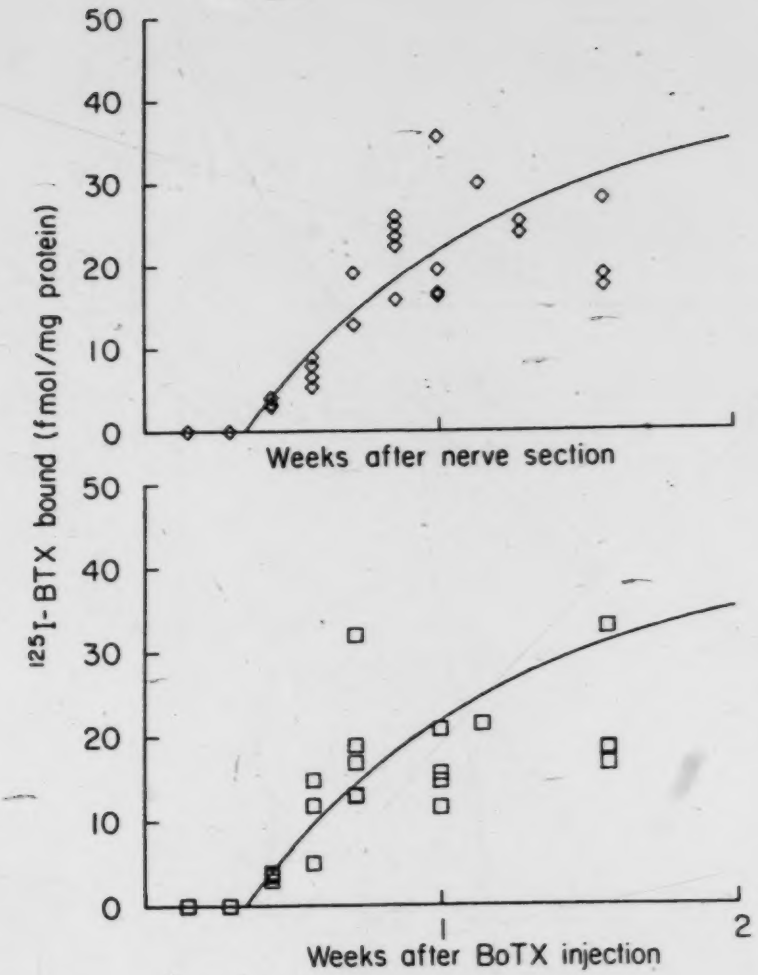


Fig. 3.6. Specific ^{125}I-BTX binding as a function of time, A. after sciatic nerve section (◇) and B. BoTX injection over the first 11 days when BoTX paralysis is complete (□). The exponential curve fitted to the data from Fig. 3.4 has been plotted in A and B. The data for the BoTX treated muscles did not deviate significantly more from the curve than the data for the denervated muscles ($p>0.05$).

complete neuromuscular blockade, BoTX treatment and denervation are equivalent in their effects on ACh receptor numbers and equivalent in the time course of this effect. Since BoTX and denervation induce comparable decreases in muscle fibre diameter; normalization of ^{125}I-BTX binding site numbers by muscle surface area again showed that nerve section and BoTX blockade produce equivalent increases in muscle ^{125}I-BTX binding site density.

3.2.3 ^{3}H-STX binding in denervated and BoTX-treated muscles. Specific ^{3}H-STX binding to crude membrane fraction or homogenates of hindlimb muscles was assayed in normal, denervated and BoTX-treated muscle. Fig. 3.7 shows that an additional 100,000g centrifugation step in the preparation of the membrane fraction produced a two-fold enrichment of ^{3}H-STX binding sites/mg protein with no change in the affinity of the site for the toxin, as measured by the Kd. However, when the total number of binding sites per mg muscle wet weight was calculated, the additional purification was accompanied by a decreased recovery of ^{3}H-STX binding sites amounting to between 40 and 60 % of the number in muscle homogenates. This reduced and variable recovery in the membrane preparations contributed to the greater scatter in the numbers of sites determined in assays using this preparation as compared to the scatter for assays using muscle homogenate.

Specific binding of ^{3}H-STX by muscle homogenate and

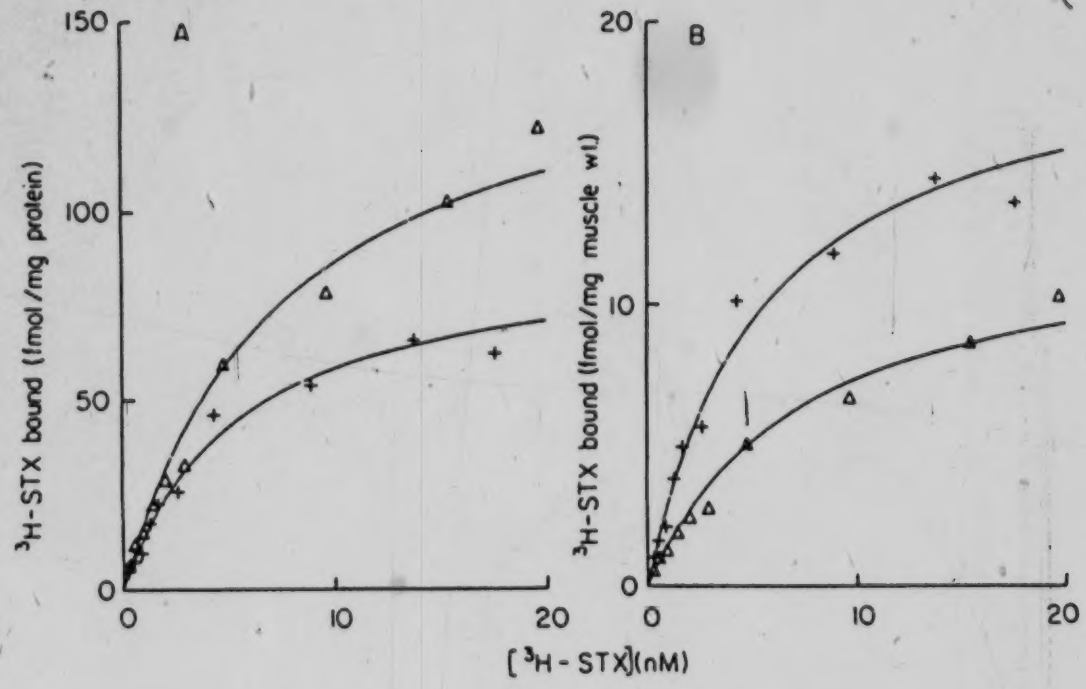


Fig. 3.7. ^{3}H-STX binding in muscle homogenate (+) and in crude membrane fractions (Δ) from normal adult rat t.t. muscles, normalized by, A. muscle protein (fmol/mg protein) and B. muscle wet weight (fmol/mg wet weight). Hyperbolic curves have been fitted to the data as described in the methods.

crude membrane fractions from control, BoTX-treated and denervated t.s. muscles is compared in Fig. 3.8. The increase in specific binding with increasing concentrations of free ^{3}H-STX was very similar for all muscles and the data were equally well fitted with the same hyperbolic curve (see Chapter 2 for details of curve fitting for ^{3}H-STX binding). This demonstrated that neither BoTX-paralysis nor denervation affected the Kd or the maximum ^{3}H-STX binding, whether the binding was normalized by muscle or membrane protein.

The mean values for maximum ^{3}H-STX binding to muscle homogenates (+) and membrane fractions (Δ) are plotted in Fig. 3.9 as a function of time after denervation. The mean values for Bmax in the denervated muscle always fell within 1 standard deviation of the corresponding mean values in control muscles for periods of denervation up to 3 weeks. The same was true for ^{3}H-STX binding to muscles 5-12 days after BoTX treatment. The numbers of ^{3}H-STX binding sites assayed for muscles denervated or BoTX treated were combined for comparison with normal muscles in Table 3.1. A three-way analysis of variance showed that the means for the 3 groups of muscles were not significantly different ($p>0.05$). Neither denervation nor neuromuscular blockade influenced the numbers of ^{3}H-STX binding sites, normalized per mg protein or wet weight, in adult muscles. This is in contrast to the marked and

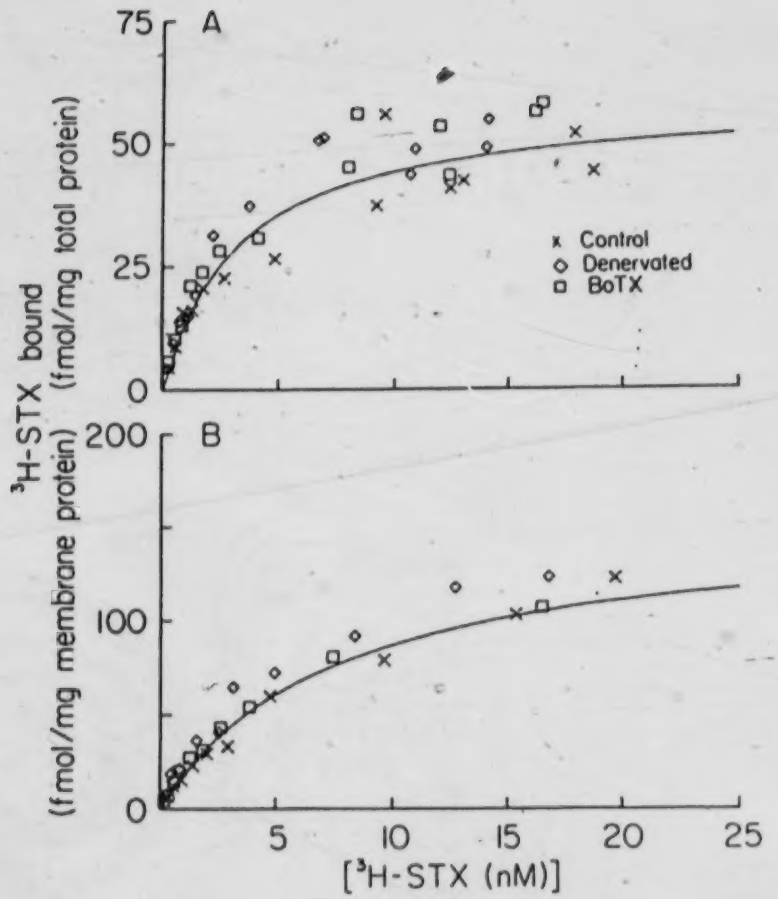


Fig. 3.8. ^{3}H-STX binding as a function of free ^{3}H-STX in, A. membrane fraction and B. homogenates prepared from normal (X), 7 day denervated (◇) and 7 day BoTX-paralyzed (□) adult muscles. The hyperbolic curve fitted to the data for normal muscle is equally well fitted through the data for the treated muscles.

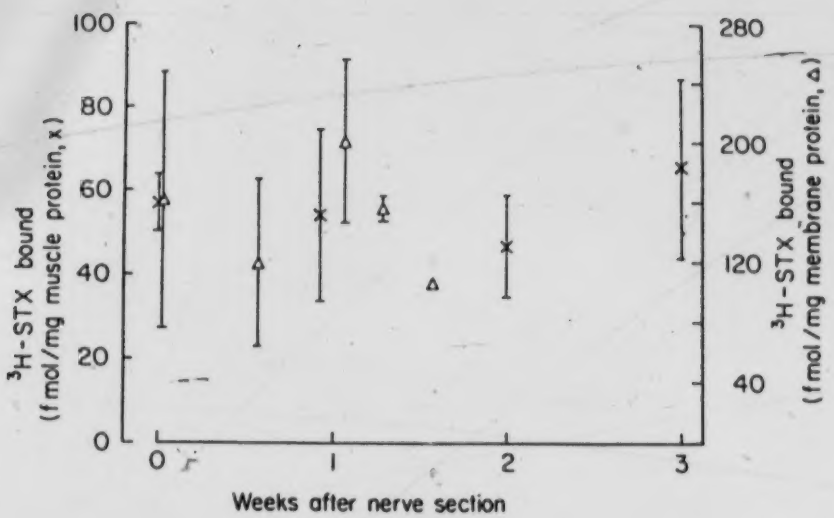


Fig. 3.9. Comparison of ^{3}H-STX binding (Mean ± SD) to muscle homogenate (△, Y-axis shown on left) and to a membrane fraction (+ ,Y-axis shown on the right) prepared from adult rat t.s. muscle as a function of time after sciatic nerve section. Note that the mean number of ^{3}H-STX binding sites in denervated muscles was not significantly different from normal values over a 3 week period after denervation. The fitted regression line for toxin binding to either muscle preparation as a function of time after nerve section was not significantly different from zero (RO=0.2, not shown). Mean values were averages of Bmax values obtained from Scatchard plots of specific binding to muscle preparations from 3-6 animals.

^{3}H-Saxitoxin Binding

	Control	Denervated	BOTX
fmol/mg Membrane protein	162.0±32(8)	152.0±19(7)	124.0±20(9)
fmol/mg Muscle Protein	57.3±3(7)	54.0±4(12)	48.0±4(9)
fmol/mg Muscle weight	7.8±1.3(7)	9.2±1.1(11)	7.7±.6(9)

Table 3 | Means ± SE (n) for the Bmax values from Scatchard plots of ^{3}H-STX binding to crude membrane fractions and muscle homogenates prepared from normal, denervated and BoTX paralyzed hindlimb muscle. Binding sites are expressed as fmol/mg membrane protein, fmol/mg muscle protein or as fmol/mg muscle wet weight. A three way analysis of the variance among control, denervated and BoTX treated muscles show no significant difference ($p>0.05$) for ^{3}H-STX binding sites in membrane fractions of homogenates.

equivalent effect of these treatments in increasing ^{125}I-BTX binding (see above) as well as to their effect in reducing ^{3}H-STX binding site numbers, relative to muscle protein or weight, in neonatal rat muscle (Chapter 5).

The actual density of toxin sites in the muscle membrane will depend on the relationships between muscle volume and both sarcolemmal and t-tubular surface areas, since up to 50% of the ^{3}H-STX binding sites may be in the t-tubular membrane (Jaimovich et al., 1976; 1983). These relationships change with muscle atrophy and the decrease in fibre diameter, both of which are similar in denervated and BoTX-treated muscles (Fig. 3.1 and section 3.2.1). The relationships between neurotoxin binding site density, muscle weight and membrane (sarcolemma and t-tubule) area are considered in detail in the discussion.

3.3 DISCUSSION

3.3.1 Muscle ACh receptors. Assays of ACh receptors in this study showed that the effects of denervation could be mimicked by blocking neuromuscular transmission with BoTX. The increases in ACh receptor numbers after denervation or presynaptic blockade of neuromuscular transmission were equivalent and followed the same time course.

The first measurable increase in ^{125}I-BTX binding occurred 3 days after either denervation or BoTX-treatment. This delay in the appearance of increased ^{125}I-BTX binding may be partially accounted for by a delay in the cessation of ACh release after treatment. Complete

BoTX blockade of neuromuscular transmission occurs only after 18-24 hours. As well, ACh may continue to be released from nerve terminals for up to 24 hours after nerve section. The lag may also be due in part to early increases in ^{125}I-BTX binding being below limits of detection of changes in ^{125}I-BTX binding in these assays (about 3 fmols ^{125}I-BTX bound/mg muscle protein). As a result of the uncertainty in determining the exact time of cessation of ACh release and the onset of increase in ^{125}I-BTX binding, it is neither possible to determine the exact duration of the lag nor to assign a physiological meaning to the observed lag.

The finding that the maximum numbers of ^{125}I-BTX binding sites in BoTX-treated rat skeletal muscle were the same as in denervated muscles confirms the conclusions made by Brown et al. (1982) from comparisons of ACh contractures in similarly treated mouse muscles. They are in general agreement with those of other investigators on the effects of pharmacological blockade of neuromuscular transmission, but there has been considerable disagreement as to whether blockade can quantitatively mimic the effects of denervation.

Our data for presynaptic blockade with BoTX agree with data obtained in the study of Pestronk, Drachman, Stanley, Price & Griffen (1980) in which neuromuscular transmission was completely blocked postsynaptically with BTX. Results from studies in which neuromuscular blockade with BoTX has

produced incomplete denervation-like effects on ACh receptors (Pestronk et al., 1976; Mathers & Thesleff, 1978) have been used as evidence that the nerve may release a neurotrophic factor other than ACh which continues to control ACh receptor synthesis in the paralysed muscles. An alternative suggestion has been that spontaneously released ACh, not blocked by BoTX, may act as a trophic agent. These reported differences in the effects of BoTX may have arisen from the experimental problem of maintaining complete neuromuscular blockade long enough to record the maximum effect on ACh receptor incorporation into the inactive muscle membrane, rather than from any inability of BoTX to completely mimic the effects of denervation. The maximum incorporation of ACh receptors into the denervated muscle membranes was reached considerably later than the time for which any study has been able to experimentaly maintain drug induced neuromuscular blockade (Fig. 3.4). The full time course of the changes in ^{125}I-BTX binding in muscles after nerve section was described by a single exponential. Using this calculated curve, we were able to show that the incorporation of ACh receptors into BoTX-treated muscles over the steepest portion of the exponential curve equalled the incorporation into denervated muscle membranes. Therefore, by extrapolation, muscles which are completely inactive incorporate as many ACh receptors as denervated muscle. These findings together with the

observation that recovery of neuromuscular transmission is associated with a decrease in ^{125}I-BTX sites 14-21 days after BoTX injection argue that the synthesis and incorporation of extrajunctional ACh receptors is suppressed by neuromuscular activity. The relative roles of ACh and electrical activity of the muscle membrane in this regulation are still debatable (cf. Mathers & Thesleff, 1978; Brown et al., 1982).

We found that the numbers of ^{125}I-BTX sites increased with the same time course in BoTX-treated and denervated muscles. However, in contractile studies of extensor digitorum longus (EDL) and soleus muscles, ACh sensitivity in BoTX-paralyzed muscles and denervated muscles became equivalent only after 9 days, with ACh sensitivity developing more slowly in the BoTX-treated muscles (Brown et al., 1982). Yet in another study the onset of increased ACh sensitivity in the BoTX treated muscles (Tonge, 1974) was similar to the onset of measurable ^{125}I-BTX binding in this study. The early rate of increase in ^{125}I-BTX binding after BoTX poisoning reported here resembles that seen after 3 to 5 days post synaptic blockade with curare (Berg & Hall, 1975) or BTX (Pestronk et al., 1980). The results taken together suggest that, if neuromuscular transmission is effectively blocked either post- or presynaptically, this loss of neuromuscular activity is equivalent to denervation in inducing ACh receptor synthesis.

3.3.2 Muscle Na channels. Denervation also alters the muscle Na channel such that up to 30% of the inward Na current in denervated muscles becomes insensitive to nanomolar concentrations of TTX (Pappone, 1980) leaving only 70% of the net current conducted through electrophysiologically normal channels. Nevertheless, in marked contrast to the plasticity of the ACh receptor in adult muscle, the number of Na channels appeared stable. Neither denervation nor neuromuscular blockade altered the number of ^{3}H-STX binding sites in homogenate or crude membrane fractions of adult rat muscle. These findings are consistent with the insignificant decline reported for ^{3}H-STX binding to sections of denervated rat hindlimb muscles (Hansen-Bay & Strichartz, 1980). Ritchie & Rogart (1977), using rat hemi-diaphragm, also concluded that denervation causes no real changes in the density of ^{3}H-STX binding sites. However Barchi & Weigle (1979), using a highly purified sarcolemma preparation, with 56-fold enrichment of ^{3}H-STX binding (Barchi et al., 1979), reported a 43% decrease in the number of ^{3}H-STX binding sites per mg membrane protein from 7.7 ± 0.07 to 4.4 ± 1.2 pmol/mg protein 10-14 days after denervation.

Our crude membrane fraction preparations yielded a two-fold enrichment of ^{3}H-STX binding sites per mg protein as compared with the membrane homogenate (Fig. 3.7). In neither preparation does ^{3}H-STX binding to denervated or BoTX-treated t.s. muscle differ significantly from toxin

binding to age matched control muscles (Fig. 3.8). When the Bmax values from individual binding assays to crude membrane muscle fractions or homogenates are examined as a function of days after denervation (Fig. 3.9), it can again be seen that denervation does not significantly change ^{3}H-STX binding site numbers expressed per mg muscle protein.

The finding that after denervation up to 30% of inward Na current was insensitive to nM concentrations of TTX (Pappone, 1980) together with direct demonstration of low affinity ^{3}H-STX sites in denervated muscle (Rogart & Regan, 1985) indicates that the low affinity ^{3}H-STX binding sites, characteristic of embryonic muscle (Frelin et al.,1984) are incorporated into adult sarcolemma after denervation. These lower affinity sites may be incorporated into the muscle membrane in addition to or as replacements for the higher affinity sites. Our results would seem to favour the first hypothesis.

Before reaching this conclusion, two possibilities must be considered. Firstly, it is possible that a decline of 30% in the numbers of high affinity ^{3}H-STX binding sites is below the resolution of the methods used here. Secondly, the comparisons of the estimates of binding site numbers are complicated by muscle atrophy in denervated and disused muscles. Considering the first hypothesis: if the numbers of high affinity binding sites were reduced down to 70% of normal values, the number of sites in

denervated muscle homogenates would be between 35 and 45 fmol/mg protein, taking into account one standard deviation of the mean. The mean of 54±4 fmol/mg protein found for denervated muscle is significantly higher than 70% of the control mean ($p<0.05$), and within the normal range. This also applies when the number of binding sites is normalized by the muscle weight. Thus a change of 30% would readily be seen with our methods of radioligand binding.

The second possibility, however, requires more consideration. After nerve section, muscle fibre diameter falls (Section 3.2.1 and Gutmann, 1948; Pellegrino & Franzini, 1963; Engel & Stonnington, 1974). as do muscle weight and protein content (Figs. 3.1-3.3 and Metafora et al., 1980). The latter two parameters are more closely correlated with muscle volume than with muscle surface area. As muscle fibre diameter declines after denervation, muscle volume decreases with the square of muscle radius and surface area decreases directly with muscle radius. Normalization of neurotoxin binding site numbers by muscle protein or wet weight will therefore overestimate the actual density of ^{3}H-STX binding sites in the membrane.

The extent of this overestimation is complicated by the location of a significant portion of the muscle Na channels in the t-tubular network (Jaimovitch et al., 1976; 1983). The relative amount of t-tubular membrane in mammalian muscle fibres increases after denervation as

measured by the number of triad formations per cross-sectional area (De Coster et al., 1985) and by the ratio of sarcotubular area to muscle volume (Engel & Stonnington, 1974). If we assume that a) 50% of the ^{3}H-STX binding sites reside in the t-tubules, as reported by Jaimovitch et al. (1976) for frog sartorius muscle; b) our assay does not discriminate between sarcolemmal and t-tubular ^{3}H-STX binding sites and c) that both populations of sites are similarly regulated following denervation, the following calculations can be made. Fourteen days after the denervation of rat gastrocnemius muscle, mean fibre area has been reported to decline 46% with an increase in surface area:volume ratio of 1.36. The sarcotubular area:volume ratio increases to 1.7, although this is an average because sarcotubular volume varies with fibre type (Engel & Stonnington, 1974). If the density of ^{3}H-STX binding sites remains constant after denervation. the number of sites per mg wet weight would have to increase by 36/2 + 70/2 or 53%. Such an increase would have been detected in our study but was not. (The 10-20 % decrease in muscle fibre diameter measured in this study one week after nerve section or BoTX treatment are in agreement with other histological studies of denervated muscle (Lomo & Rosenthal, 1972; Engel & Stonnington, 1974)).

Our data and that of others for denervated and disused muscles, showing no significant change in binding site

numbers relative to protein or muscle weight, would be consistent with a decrease in the density of Na channels in the membrane, as shown by Barchi & Weigle (1979) in a highly purified membrane fraction. Although we found a small but insignificant decline in numbers of ^{3}H-STX binding sites in our very crude membrane fraction, the variability encountered as a result of variable yields may have obscured a real decline. Since BoTX-treatment caused a reduction in muscle weight, protein and fibre diameter which was similar to muscle denervation, these results would also indicate that the effects of BoTX-treatment and muscle denervation on the density of the high-affinity ^{3}H-STX sites are not distinguishable.

We therefore conclude that our data are consistent with a decline in density of the high-affinity ^{3}H-STX sites in the muscle membrane after denervation or BoTX-treatment and that these high-affinity sites may be replaced by the TTX-insensitive, low toxin affinity channels described by Pappone (1980) and Rogart & Regan (1985). Alternatively, denervation and muscle inactivity may induce a transformation of Na channels *in situ* from the high to a low affinity state.

CHAPTER FOUR: A COMPARISON OF THE EFFECTS OF DENERVATION AND MOTOR UNIT ISOLATION ON HINDLIMB MUSCLES OF THE CAT: CHEMOSENSITIVITY AND MEMBRANE STABILITY.

4.1 Introduction

A number of different experimental measures have been used to render muscles inactive in order to study the role of neuromuscular activity in regulating muscle properties. Chemical means, such as neuromuscular blockade with BoTX or conduction block with TTX (Lavoie et al., 1977; Mathers & Thesleff, 1978; Brown et al.,1982; St.-Pierre & Gardiner, 1985), and physical means, using leg braces or tail suspension (Fournier et al., 1983; Gupta et al., 1985), have been used to render the muscle inactive.

Both of these methods have limitations. Although the chemical blockers are effective, they are not easily employed for long term - months to years - studies. As was seen in Chapter 3, BoTX blockade of neuromuscular transmission could not be maintained for more than 11-14 days and although the early time courses of muscle changes following denervation or inactivity were the same, the data had to be extrapolated to infer that the effects of BoTX and denervation were, in fact, equivalent. Therefore, in order to assess the role of activity in mediating nerve-muscle interactions, long term blockade of activity is required. This is because the time courses of many denervation-induced effects may be longer than 1-2 weeks. In addition, products of nerve degeneration may induce

initial changes in the muscle and therefore, accurate assessment of the importance of activity requires that inactivity should be maintained for at least 10-14 days, by which time nerve degeneration is completed. As well, the use of blockers of ACh does not permit a discrimination between a neuronal control of muscle mediated by muscle activity or by ACh.

The methods involving physical immobilization of a muscle are unsatisfactory for such studies because, although the muscle can be immobilized for long periods of time, muscle activity may not be completely abolished. Thus the results of such experiments were not unambiguous since differences between immobilized and denervated muscles could have been due to residual muscle activity, rather than to a difference between the effects of complete inactivity and denervation.

The technique of spinal isolation (Tower, 1937) can be used for long term studies of the regulation of muscle properties by activity. In particular, in conjunction with work presented in Chapter 3 on the effects of BoTX blockade in adult rat muscles, the experiments presented here on spinal isolation of cat motor units will permit an assessment of the possible role of spontaneous ACh release in nerve-muscle interactions; since BoTX does affect spontaneous ACh release to some extent while spinal isolation does not. In the modified procedure used here (Eldridge, 1984b) the spinalized animals showed a total

flaccid paralysis of the hindlimbs, with complete survival of motoneurons (Liebhold & Eldridge, 1981), for periods of up to 3 years (Eldridge et al., 1981).

Previous work on cat hindlimb muscles innervated by spinally isolated motoneurons had demonstrated that by 2-3 years after the operation, extrajunctional ACh receptor numbers, although much lower than found in 3-10 month denervated muscles, were still elevated above control values (Eldridge et al., 1981). However, direct comparisons were not made between muscles denervated or inactivated by spinal isolation for the same periods of time.

Following denervation, muscle membranes become less stable and positive sharp waves and fibrillation potentials appear. Similar changes in membrane stability have also been reported following spinal isolation of the motor units (Eldridge & Mommaerts, 1984). Interestingly, although these changes appeared at the same time in denervated and isolated muscles, fibrillation potentials were no longer found in spinal isolation muscles by 2 months after surgery. In conjunction with the equivalent early effects of denervation and BoTX-induced muscle inactivity reported earlier (Chapter 3), this suggests that muscle inactivity may be more effective in initiating that in maintaining denervation-like changes in muscle properties.

In this study, the effects of spinal isolation and

denervation (by ventral root section) on ACh receptors, muscle weight and the occurrence of fibrillation potentials were compared 3 weeks and 8 months after surgery. ACh receptors were therefore measured when the inactive and denervated muscles were both fibrillating and later, when the membrane stability of the spinal isolation muscles was re-established. Thus, the possibility of a biphasic regulation of extrajunctional ACh receptors by inactivity was examined and the regulation of ACh receptors was investigated at the time when another aspect of the membrane function was changing. Assessment of ^{125}I-BTX binding in 3 week and 8 month denervated muscle allowed, in conjunction with the results of Chapter 3, the description of a more complete time course of the effects of nerve section on muscle ACh receptor numbers in order to verify our earlier finding that maximum ACh receptors numbers wre expressed by 3 weeks after nerve section (Chapter 3).

These experiments demonstrated that spinal isolation of the motor unit did increase numbers of ^{125}I-BTX binding sites in cat hindlimb muscles. After 3 weeks the effect was, in some muscles, equivalent to denervation. By 8 months, the number of sites in the quiescent muscles had recovered markedly back towards control levels although no recovery of muscle function was observed. It is suggested that membrane depolarization consequent to non-evoked release of ACh may play a role in the regulation of muscle

ACh receptors. It was also found that ^{125}I-BTX binding site numbers in 3 week and 8 month denervated cat hindlimb muscles were not significantly different. This confirmed and extended the earlier result for rat hindlimb muscles that maximal numbers of ^{125}I-BTX binding sites were incorporated by 3 weeks after nerve section.

4.2 RESULTS

4.2.1 Effectiveness of nerve section and spinal isolation. Ventral root section and spinal isolation of the motor units resulted in complete flaccid paralysis of the hindlimb muscles as described in the methods (Chapter 2). This flaccid paralysis persisted unaltered during the entire 8 months. In a previous study (Eldridge et al., 1981) paralysis was maintained for up to 3 years after the spinal isolation operation.

4.2.2 ^{125}I-BTX binding to hindlimb muscles. As was the case with rat muscle, specific ^{125}I-BTX binding was not measurable in normal adult cat muscle since values were below the lower limit of detection (about 3 fmol/mg protein). Following denervation, ACh receptor numbers increased in gastrocnemius, plantaris, and soleus muscles (Fig. 4.1). ^{125}I-BTX binding was higher in cat than in rat hindlimb muscles denervated for the same period of time (90-100 fmol/mg protein as compared to 39 fmol/mg protein at 3 weeks, see Chapter 3), probabely reflecting a higher extrajunctional density of ACh receptors in denervated cat, as compared to rat, muscle.

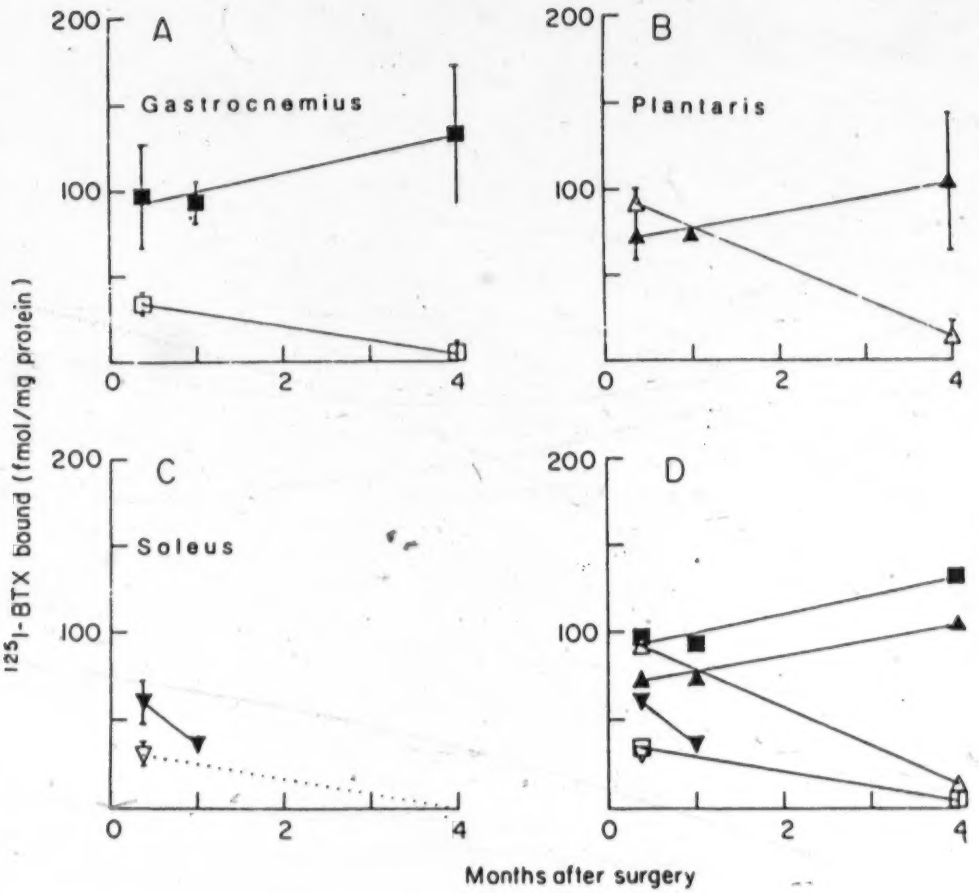


Fig. 4.1. Specific ^{125}I-BTX binding to gastrocnemius (A), plantaris (B) and soleus (C) muscles as a function of time after root section (closed symbols) or spinal isolation of the motor units (open symbols). Data are means +SE for 2-6 muscles. The collation of the data in (D) shows that ^{125}I-BTX binding does not increase significantly between 3 weeks and 8 months after root section (slope of the regression line not significantly different from zero, $p>0.05$) but the binding decreases to near control values for spinal isolation muscles. Dotted line in C is predicted time course for ^{125}I-BTX binding in soleus muscles of spinal isolation cats based on results for gastrocnemius (A) and plantaris (B).

The effectiveness of denervation at the root level in elevating ACh receptor numbers was equivalent at 3 weeks to that of denervation at the sciatic level at 2 months (Fig. 4.1). This is in agreement with a report by Steinbach (1981) who did not observe any systematic difference in effectiveness between the two treatments.

^{125}I-BTX binding was elevated above control values by 3 weeks after ventral root section and these elevated values were maintained through 2 and 8 months after nerve section. This is shown in Fig.4.1 (A,B) for gastrocnemius and plantaris muscles where the slopes of the lines calculated for the numbers of ^{125}I-BTX binding sites as a function of time after treatment were not significantly different from zero. This is in agreement with the results obtained for adult rat muscles (Chapter 3) where maximum numbers of ACh receptors were incorporated by 3 weeks after nerve section.

Muscle inactivation by isolation of the motoneurons from supraspinal and segmental inputs also induced increased synthesis and incorporation of extrajunctional ACh receptors in all 3 cat hindlimb muscles studied (Fig. 4.1). In fact, spinal isolation and denervation were equally effective in increasing ^{125}I-BTX binding in plantaris and soleus muscles ($p>0.05$), although the effect of spinal isolation fell short of denervation in gastrocnemius muscles. This increased ^{125}I-BTX binding was not maintained in muscles of isolated motor units and

toxin binding had declined towards normal levels by 8 months after the spinal isolation operation while ^{125}I-BTX binding remained high in the denervated muscles. Silencing the intact motor units is, therefore, effective in inducing an initial increase in muscle ACh receptor numbers but, in contrast to the persistent effect of denervation, these elevated numbers of ACh receptors are not maintained.

During the normal development of skeletal muscle, numbers of extrajunctional ACh receptors decrease. This decrease in ACh receptor numbers can be inhibited by denervation or inactivity and the magnitude of the increase in ACh receptor numbers is greater following denervation or inactivity in developing as compared to adult muscles (Chapter 5). Although a developmental study of ^{125}I-BTX binding in cats has not been undertaken here, in one kitten (4 months old), studied 22 days after the spinal isolation operation, ^{125}I-BTX binding was 45 and 42 fmol/mg protein for the right and left t.s muscles (gastrocnemius and plantaris together), respectively. This is slightly, but not significantly, higher than the values for adult muscles similarly treated. Muscle atrophy was also similar, the soleus muscle being 0.57 g/kg of cat body weight at death (cf. Fig. 4.2).

4.2.3 Muscle membrane stability. Fibrillation potentials were first observed in cat hindlimb muscles 1-2 weeks after denervation or spinal isolation of cat muscles.

These potentials were evident in all denervated muscles throughout the 8 month period. In contrast, in muscles of the inactive motor units of the spinal isolation cats, fibrillation potentials continued to be present for only 3-5 week after commencing and fibrillation potentials were no longer present 5-7 weeks after surgery (Fig. 4.2 and Eldridge & Mommaerts, 1984).

4.2.4 Muscle atrophy. Adult cats (aged 2-4 years) used in this study ranged in weight from 2.1 to 4.8 kg at the time of surgery. The average weight was 3.5±0.3 kg (X±SE; n=10). For comparison of individual hindlimb muscle weights, the muscle weight was expressed as g per kg cat weight. Because cat body weight was sometimes reduced by hindquarter wasting in the 8 month root section animals, pre-surgery weight was regularly used as the normalizing factor.

Normalized muscle weights are shown in Fig. 4.2 as the means of 2-6 muscles from 1-3 cats. All 3 muscles measured, plantaris, medial gastrocnemius and soleus, decreased in weight following denervation or spinal isolation. This decrease is progressive with time, all muscles weighed less after 8 months than after 3 weeks. The muscle atrophy induced by spinal isolation of the innervating motoneurons is less similar to denervation for gastrocnemius as compared to plantaris and soleus muscles, similar to the difference in the effectiveness of spinal isolation in increasing ^{125}I-BTX binding in the 3 hindlimb

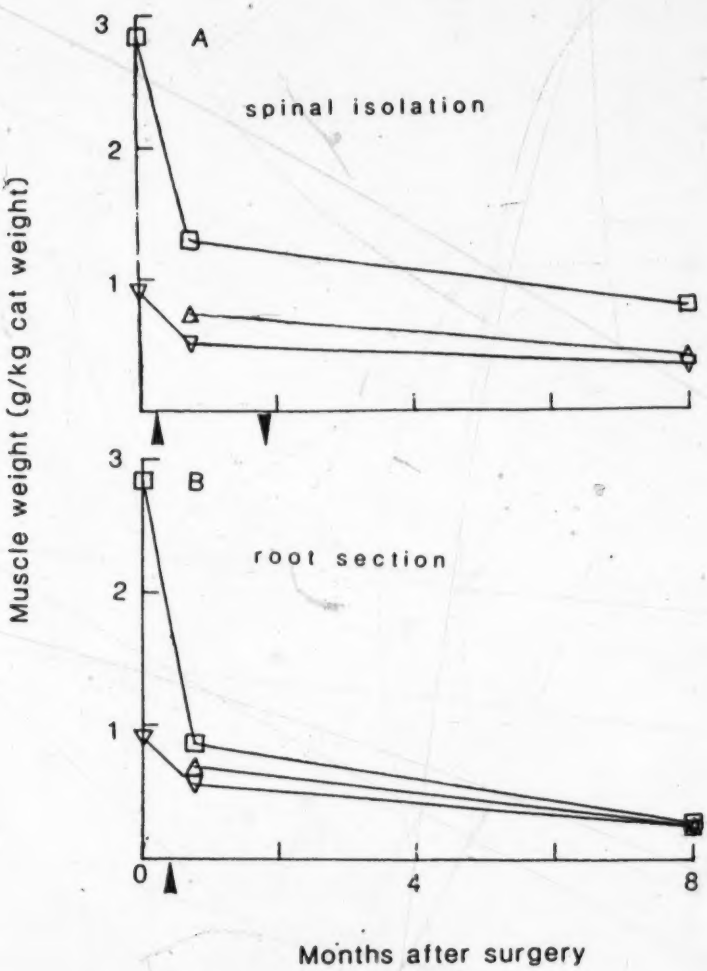


Fig. 4.2. Decrease in medial gastrocnemius (□), soleus (▽) and plantaris (△) weights (g/kg cat weight) as a function of time after A. spinal isolation of the motor units or B. ventral root section. Control muscle weights were taken, by permission, from work of R. Orozco. Data shown are the means of 4-6 muscles. Time when fibrillation potentials were first observed. ▲ Time after which fibrillation potentials were no longer observed. ▼

muscles (section 4.2.2).

4.3 DISCUSSION

Both denervation and isolation of intact motor units induced the appearance of extrajunctional ACh receptors, fibrillation potentials and muscle atrophy for cat hindlimb muscles. The increase in ACh receptor numbers and the decrease in membrane stability were maintained in denervated but not in isolated hindlimb muscles. Muscle atrophy persisted following either treatment but was more severe after denervation. Therefore, the loss of activity in the intact but silent motor units of the spinal isolation cats was sufficient to induce the appearance of denervation-like changes in the cat muscles. However, induction and maintenance of these changes appear to be differently regulated as inactive muscles begin to recover normal muscle properties by 8 months, when there was still no recovery of muscle activity.

With respect to the increase in ACh receptor numbers and decrease in muscle weight, soleus and plantaris muscles were more affected by inactivity than was gastrocnemius. This agrees with a reports of a more pronounced and rapid effect of BoTX-induced inactivity on soleus as compared to gastrocnemius muscle (Duchen, 1970; 1971b) The soleus is a postural muscle and more frequently activated than gastrocnemius (Vrbova, 1963). Soleus muscle may be more dependent on this activity for maintenance of its properties than the less active gastrocnemius muscles

and this may be why the loss of activity consequent on spinal isolation affects soleus more than gastrocnemius. Again, this suggests the importance of activity in regulating muscle properties.

BoTX treatment (Chapter 3) was more effective than spinal isolation in mimicking the effects of denervation in increasing ACh receptor numbers. Muscles in both chemically and surgically treated animals were inactive and evoked release of ACh was inhibited in both BoTX-treated and isolated motoneurons, but only in the BoTX-treated muscles was non-evoked release of ACh also inhibited (Dolezal et al., 1983; Gunderson & Jenden, 1983; Stanley & Drachman, 1983). Therefore, comparison of the effects of BoTX-paralysis on ACh receptor numbers in rat t.s. and the effects of spinal isolation of gastrocnemius muscle in cats, suggests that the non-evoked release of ACh may play a role in regulating muscle properties.

Acetylcholine present at the silent neuromuscular junctions of the spinal isolation cats could be influencing ACh receptor synthesis by inducing sub-threshold depolarizations (Gruener, Baumbach & Coffee, 1974) of the muscle membrane consequent on activation of the nicotinic ACh receptor. This hypothesis is attractive as it may provide an explanation for the recovery of muscle properties in the isolated motor units. The effectiveness of spontaneously released ACh at the silenced muscle fibres in causing local depolarizations of

the muscle membrane would increase with continued muscle disuse since muscle membrane input resistence increases as muscle fibre diameter declines and the muscle atrophies (Katz & Thesleff, 1957). Thus, since the magnitude of the non-evoked ACh induced depolarizations increases with time, ACh-mediated inhibition of ACh receptor incorporation would also increase. The differences between plantaris and soleus on the one hand and gastrocnemius on the other, may reflect different quantities of spontaneously released ACh present at the different inactive muscles or a different relative importance of the evoked and non-evoked release of ACh in regulating ACh receptor synthesis and incorporation in the different muscle membranes.

It is also possible that the regulation of ACh receptor synthesis by activity may be mediated by some effect of the interaction of ACh with the nicotinic ionophore other than the membrane depolarization or by an increased release of some other trophic substance. In either case, the regulation may be mediated by second messengers. Intracellular Ca levels are affected by muscle activity and previous reports (discussed in Chapter 6) have shown that Ca may be the intracellular mediator for the regulation of ACh receptor synthesis by muscle activity.

Another possible explanation for the recovery of muscle properties towards normal values in the spinally isolated motor units is that there was some recovery of

neuromuscular activity after 1-2 months. Although no motor unit activity was recorded in the EMG, the fact that such recordings were made only intermittently and always from seated, relaxed cats, leaves open the possibility that some motor units were still activated, perhaps only during specific activities, in these cats. Chronic EMG recording would be necessary to clarify this possibility. In addition, synaptic connections within the quiescent, isolated, spinal cord may vary with prolonged isolation and the release characteristics of the inactive terminals may change. Again, chronic recording of muscle activity and muscle responses to nerve stimulation would be useful in determining the role such putative changes may be playing in the regulation of muscle properties in spinally isolated motor units.

Nevertheless, it can be concluded that, in all 3 muscles, activity does have a trophic role in regulating nerve-muscle interactions but inactivity is less effective than denervation in maintaining increased muscle ACh receptors, decreased membrane stability and muscle atrophy.

CHAPTER FIVE: THE INFLUENCE OF INNERVATION AND ACTIVITY ON THE ACETYLCHOLINE RECEPTORS AND SODIUM CHANNELS OF DEVELOPING RAT MUSCLE

5.1 INTRODUCTION

During the normal development of skeletal muscle, extrajunctional regions of the muscle fibres lose their chemosensitivity (Diamond & Miledi, 1962; Gordon & Vrbova, 1975) with the disappearance of extrajunctional ACh receptors (Fambrough & Hartzell, 1972; Bevan & Steinbach, 1977) and the restriction of ACh receptors to the endplate region. This differentiation of muscle chemosensitivity is paralleled by a change in muscle excitability, as muscle action potentials become sensitive to blockade by TTX (Harris & Marshall, 1973; McArdle et al., 1980) and the numbers of Na channels with high affinity binding sites for ^{3}H-STX increase (Sherman & Catterall, 1982). These changes are the reverse of those occurring after denervation of adult muscle. Denervation of the developing muscle halts or reverses this differentiation (Vrbova et al., 1978; Sherman & Catterall, 1982; Ziskind-Conhaim & Bennett, 1982). Neuromuscular blockade has effects similar to denervation in inhibiting the restriction of ACh sensitivity to the endplate region but the effects of such a blockade on Na channels during development have not been established.

In this study, the role of neuromuscular activity in regulating the developmental changes in both muscle ACh

receptors and Na channels was studied. It was found that the development of ^{3}H-STX binding sites in neonatal rat muscle correlated with the onset of adult patterns of neuromuscular activity and with an acceleration in muscle growth and was preceded by the decrease in ^{125}I-BTX binding site numbers. Presynaptic neuromuscular blockade with BoTX or muscle denervation halted or reversed the maturational decrease in ^{125}I-BTX and the increase in ^{3}H-STX binding, but BoTX was less effective than denervation. Possible roles for evoked and non-evoked ACh release, the products of nerve degeneration and other factors in mediating the neuronal regulation of muscle ACh receptors and Na channels are discussed.

5.2 RESULTS

5.2.1 Muscle growth.

5.2.1.1 Development. The rate of muscle growth increases between 2 and 3 weeks after birth. This is seen for muscle weight and protein (Fig.5.1) and for fibre diameter (Table 5.1). This growth spurt occurs at the time of rapid body growth (Fig. 5.1), increased muscle use and the development of adult patterns of activity in the rat hindlimb muscles (Navarrete & Vrbova, 1980; 1983; Vrbova, Navarette & Lowrie, 1985). During this time the neonatal rats' eyes open (10-11 days) and limb movement becomes more coordinated. The muscle growth spurt also correlates with the loss of polyneuronal innervation of the muscles (Redfern, 1970; Bennett & Pettigrew, 1974; Brown et al.,

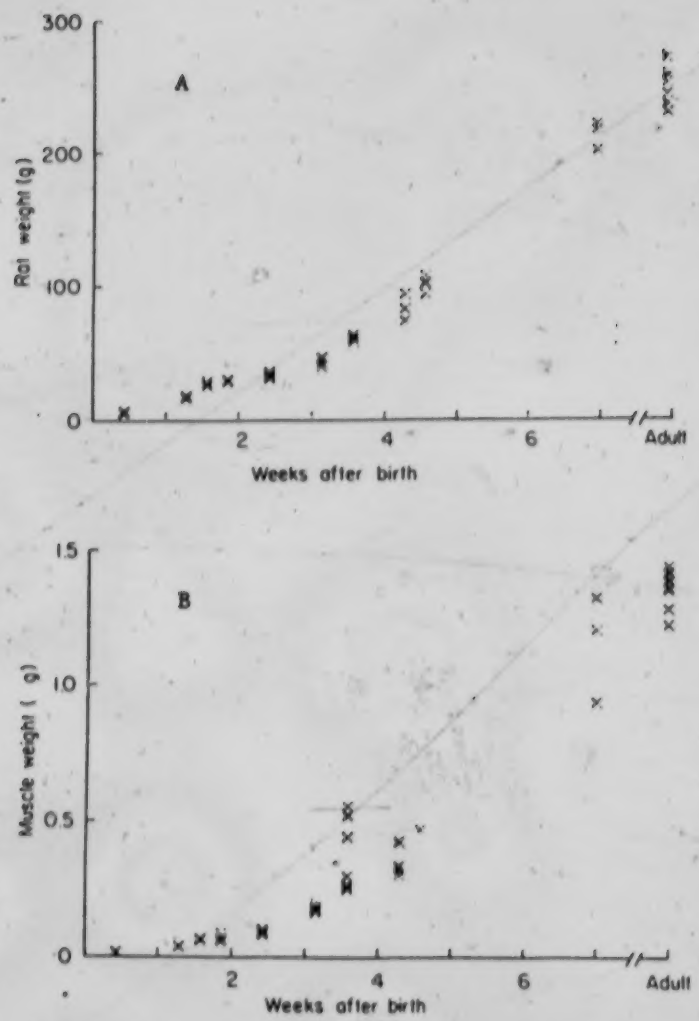
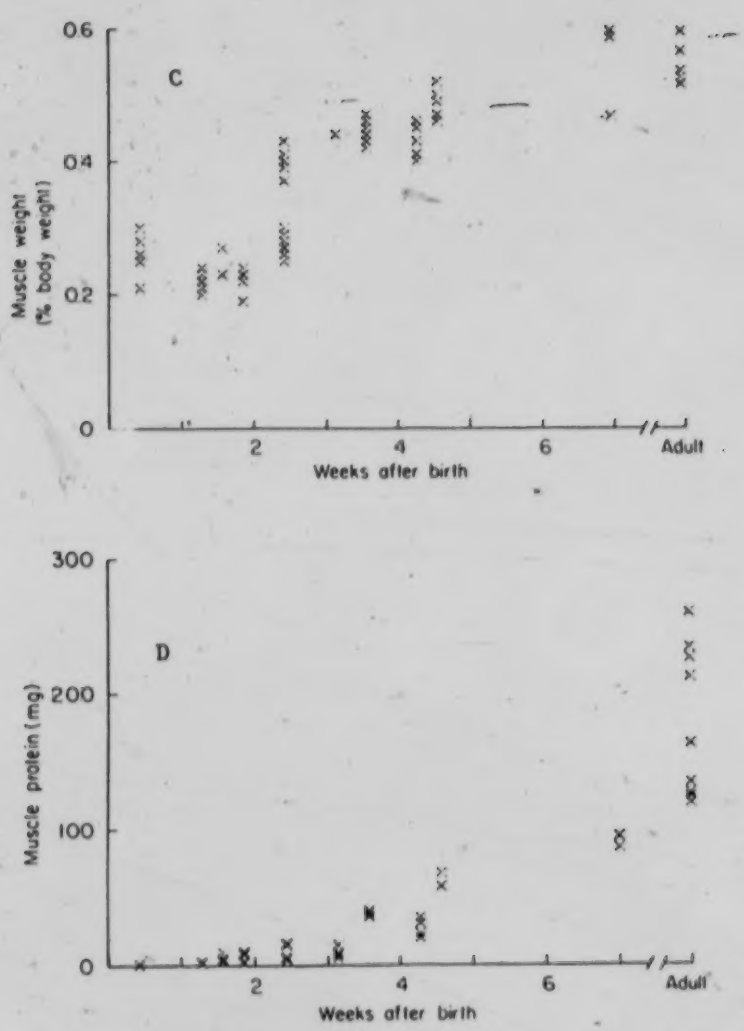


Fig. 5.1. Developmental increase in A. rat weight, B. t.s. muscle weight(grams),as a function of weeks after birth.



C. t.s. muscle weight (percentage of rat body weight) and D. t.s. muscle protein as a function of weeks after birth. Note the accelerated growth after 2-3 weeks. Data represent values for individual muscles.

Age (weeks)		Muscle weight* (mg)	Muscle % body weight*	Muscle protein* (mg)	Muscle fibre radius△ (μm)
1		38.6±0.6, 6	0.22±0.1, 6	2.5±0.3, 4	7.5±0.2, 349
2	C	93±2.0, 12	0.27±0.01, 12	11±3, 12	11.0±0.3, 248
	D	74.5±0.3, 12	0.21±0.01, 12	6±3, 6	8.0±0.2, 564
	B	61±14, 4	0.22±0.04, 4	8±4, 4	10.0±0.3, 216
3	C	225±14, 14	0.42±0.01, 14	23±5, 10	17.3+0.4, 276
	D	118±14, 6	0.22±.02, 6	18±5, 6	8.4±0.2, 310
	B	129±14, 8	0.29±0.03, 8	21±4, 8	13.4±0.6, 389
4	C	424±26, 12	0.45±0.01, 12	40±7, 8	18.7±0.4, 288
	D	224±7, 8	0.24±0.02, 8	23±4, 8	10.9±0.3, 265
	B	286±20, 8	0.36±0.02, 8	25±4, 6	14.0±0.3, 411
7	C	1373±23, 10	0.55±0.01, 10	178±19, 9	27.5±0.4, 200
	D	1008±35, 10	0.48±0.02, 10	114±9, 8	24.0±0.4, 180
	B	986±25, 10	0.46±0.03, 10	100±7, 7	22.5±0.5, 191

Table 5.1. Rat t.s. muscle weight in grams and relative to rat body weight; t.s. muscle protein and muscle fibre radius for normal t.s. muscle during development (C) and for experimental muscles during development at the same stage but one week after sciatic nerve section (D) or BoTX treatment (B). Data are expressed as mean±SE, where n=number of muscles measured (*) or n=numbers of fibres measured in a total of two muscles (△).

1976; O'Brien et al., 1978). The temporal correlation of the muscle growth spurt with changes in neuromuscular activity and muscle innervation suggests that nerve and, in particular, the activity imposed on the muscle by the nerve, may be important in the regulation of muscle growth.

5.2.1.2 Denervation and BoTX treatment. Denervation at any developmental age decreased muscle weight, protein content and fibre radius relative to age matched controls (Table 5.1). Sciatic nerve section at times earlier than 3 weeks after birth retarded but did not prevent muscle growth, measured as muscle weight and protein content. Similarly, muscles paralysed with BoTX also atrophied relative to age matched controls (Table 5.1).

When muscle weight was normalized by body weight (to control for systemic effects of the BoTX-treatment, see Chapter 8), BoTX-treated muscles were consistently less atrophied after 1 week than were the comparable denervated muscles. Similar results were obtained when muscle fibre radii were used as a measure of atrophy. Although all BoTX-treated neonatal muscles showed some signs of recovery of neuromuscular transmission by 1 week after injection (Chapter 8), incomplete paralysis did not completely account for the lesser atrophy of the toxin-treated, as compared to the denervated muscles: the lesser atrophy of BoTX-treated muscles was also evident from the significantly smaller decline in normalized muscle weight

and protein content for muscles 3 days after treatment ($p<0.05$), when the BoTX-blockade of neuromuscular transmission was complete. The lesser atrophy of disused neonatal muscles contrasts with the equivalent atrophy of BoTX-treated and denervated adult rat muscles (Chapter 3).

5.2.2 ACh receptors

5.2.2.1 Development. ACh receptors are located all along the immature muscle membrane. This high extrajunctional ACh receptor density was measured as high levels of ^{125}I-BTX binding (Fig. 5.2; 5.3). Three days after birth the numbers of ^{125}I-BTX binding sites were very high (119±14 fmol/mg protein and 4.8 fmol/mg ±0.4 fmol/mg muscle; X±SE) declining to undetectable levels in adult muscle where the ACh receptors are confined to the endplate region (Diamond & Miledi, 1962; Gordon & Vrbova, 1975; Bevan & Steinbach, 1977).

In 18 day old rat embryos, hindlimb muscle are innervated 2-4 days previously (Bennett & Pettigrew, 1974). ^{125}I-BTX binding measured in these hindlimb muscle was significantly lower than in muscles of 3 day postnatal rats (60±10 fmol/mg protein and 1.7±0.2 fmol/mg wet weight). This difference was artificial because it resulted from contamination of the muscle homogenates prepared from the fetal rats. (Bone and skin were included in the homogenization of the very small, amorphous lower limbs of the embryonic rats in contrast to the purer muscle homogenates prepared from the neonatal rats. These

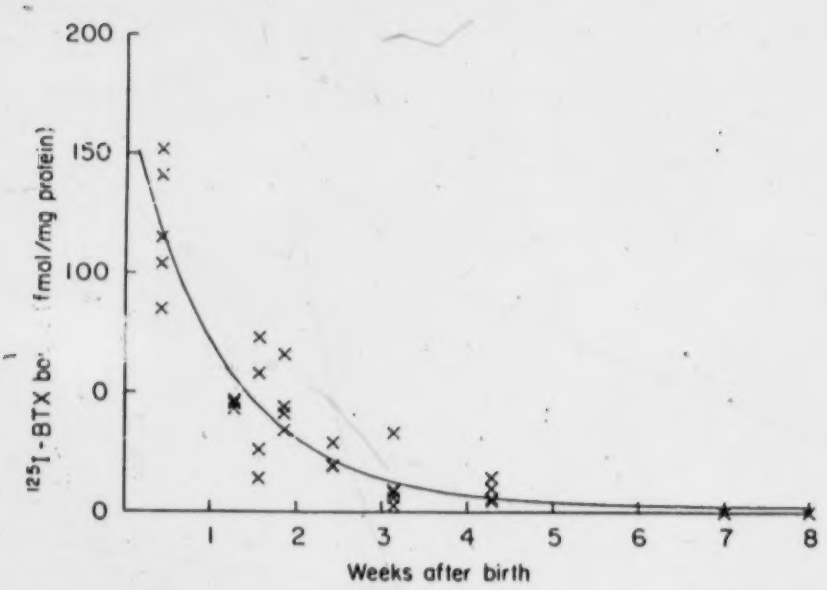


Fig. 5.2. Specific ^{125}I-BTX binding relative to muscle protein as a function of age. The binding, relative to muscle protein, decreases after birth reaching low, unmeasurable, adult levels before 7 weeks. The data have been fitted with a single exponential with a time constant of 8 days. When the data were plotted as the log of the toxin binding versus time, the relationship was linear with a correlation coefficient of 0.95 and the line was, therefore, significant at $p<0.01$ (not shown).

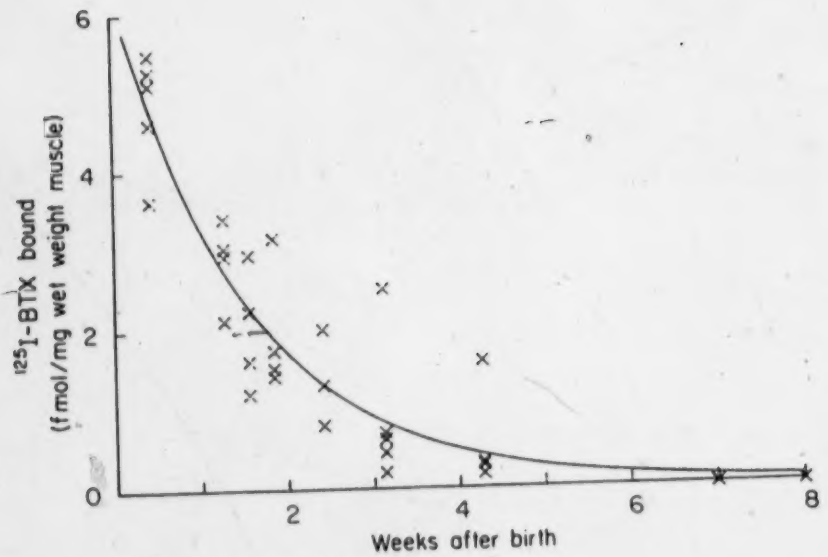


Fig. 5.3. Specific ^{125}I-BTX binding, relative to muscle wet weight, as a function of rat age. The binding decreases after birth reaching adult levels before 7 weeks. See Fig. 5.2.

contaminants were reduced to some extent by filtration but not sufficiently to reduce their contribution to both the weight and, to a lesser extent, to the protein used in normalizing the binding site data. Visual observation indicated that as much as two-thirds of the weight may have been accounted for by non-muscle components so that normalization by weight or protein would underestimate the actual number of ^{125}I-BTX binding sites by as much as a factor of 3.) Taking this contamination into account, the numbers of binding sites in the embryonic muscle can be estimated as 5.1 fmol/mg muscle which was not significantly different from the value of 4.8 fmol/mg muscle for 3 day postnatal rat hindlimb muscle. Peak levels of extrajunctional ACh receptors in the newborn rats are thus established *in utero* and are maintained at a plateau across the perinatal period, prior to their rapid decline in the first 2 weeks of life (see Figs. 5.2, 5.3). The plateau is preceded by an earlier rapid decline in ACh receptor numbers from their highest levels in muscles at 15 to 18 days *in utero*, during which time the muscle fibres become innervated (Bevan & Steinbach, 1977).

During the first 2 weeks of postnatal life, the numbers of extrajunctional ACh receptors decline rapidly. The decline in ^{125}I-BTX binding sites with time is well fitted by a single exponential for data normalized by muscle protein (Fig. 5.2) or muscle weight (Fig. 5.3) to attain low and unmeasurable adult levels with a time constant of

8 days. By 7 weeks of age, the sites were not detectable using radioligand binding techniques, the density of the adult extrajunctional ACh receptors being extremely low (<5/μm^2, see Chapters 1,3).

Comparison of the decline in levels of extrajunctional ACh receptors with the rate of muscle growth in Fig. 5.4, showed that the decrease in extrajunctional incorporation is an early postnatal event and that the most rapid decline precedes the rapid phase of muscle growth. Muscle growth, in turn, appears to correlate with the period of increased motor activity which follows eye opening (see above).

When neuromuscular activity was interrupted in the second week of life, just prior to the opening of eyes in the rat and the increase in motor activity, ACh receptors were again incorporated into the extrajunctional membrane (Fig. 5.5). The levels of ^{125}I-BTX binding sites increased and exceeded the numbers found in early neonatal muscle halting and reversing the developmental decline. Although curve were fitted by eye through the ^{125}I-BTX binding data in the Fig. 5.5, the complete time course of this increase was not established because ^{125}I-BTX binding site numbers were not assayed in muscles more than 1 week after sciatic nerve section.

Numbers of ^{125}I-BTX binding sites also increased when neuromuscular activity was blocked with BoTX. However, the effect of BoTX-treatment was not equivalent to

Fig. 5.4. Changes in mean ^{125}I-BTX binding (from Fig. 5.2) (X, reduction in ^{125}I-BTX binding relative to the average value at 3 days postnatal), t.s muscle weight in grams (▲) and t.s. muscle weight relative to rat body weight (△) (from Fig. 5.1) plotted as percentages of their respective adult values as functions of time after birth. Toxin binding had declined to near adult levels by 2 weeks when muscle weight is just beginning to increase more rapidly.

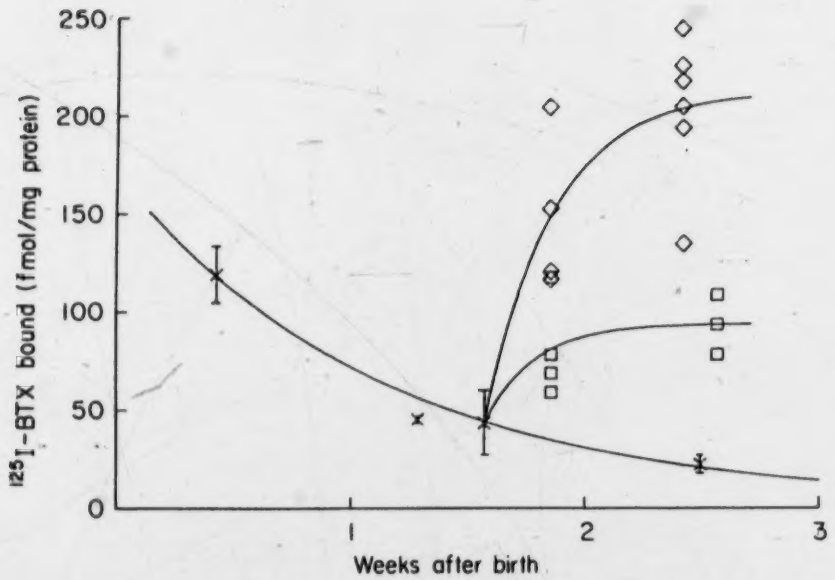


Fig. 5.5. ^{125}I-BTX binding to t.s. muscle as a function of time after sciatic nerve section (◇) or BoTX injection (□) at day 11 relative to control muscles (×) (mean± SE from Fig. 5.2). Values for treated muscles are the means of 3-4 replicate determinations for homogenates from single muscles. Note the greater effect of denervation at 3 days and 1 week after treatment.

denervation. Incomplete neuromuscular blockade with BoTX at 1 week after injection was not the entire reason for the unequal effects of BoTX and denervation since when BoTX-blockade was complete, at 3 days, ^{125}I-BTX binding was still lower in the paralyzed than in the denervated muscles. This contrasts the equivalent effects of denervation and BoTX-blockade in increasing ^{125}I-BTX binding in adult muscle.

As shown in Figure 5.6 denervation elevated ^{125}I-BTX binding at all developmental stages above age matched controls and above control values at the time of surgery. Therefore, denervation reversed, rather than halted, the decrease in numbers of ACh receptors which normally occurs during muscle maturation in neonatal life.

When ^{125}I-BTX binding was normalized by fibre radius to compensate for the wide difference in muscle fibre surface area:volume ratios at different developmental stages (Table 5.2), it was clear that denervation of juvenile or mature muscle increased ACh receptor density well above levels found in normally innervated postnatal rat t.s. muscle and closer to the levels found in earlier embryonic muscles shortly after innervation *in utero*. Denervation may, therefore, induce a dedifferentiation of the muscle membrane to a less mature embryonic form.

Neonatal sciatic nerve section at all developmental stages resulted in a similar 6- to 7-fold increase in ^{125}I-BTX binding site density relative to age matched

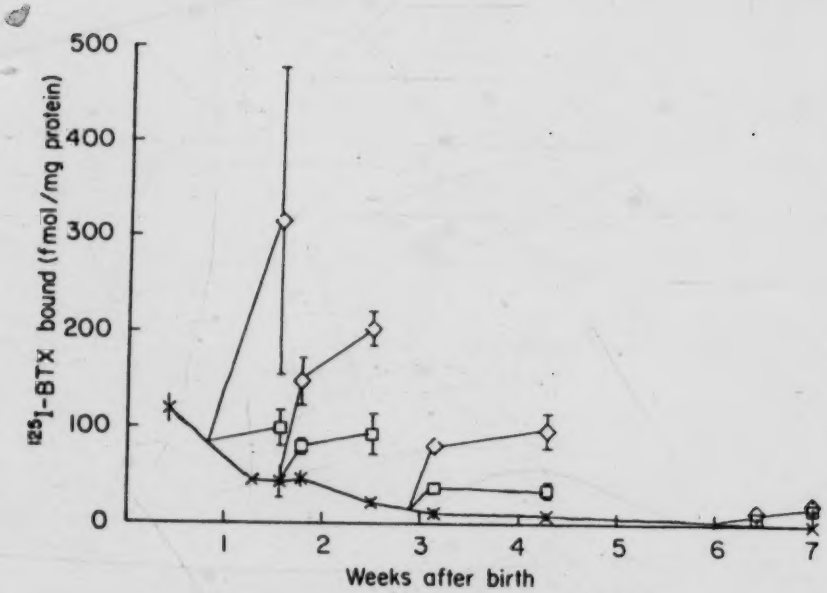


Fig. 5.6. ^{125}I-BTX binding to control t.s. muscle (X) and to t.s. muscle after sciatic nerve section (◇) or BoTX injection (□) for rats treated 1-6 weeks after birth. (mean±SE, n=4-6 muscles).

Age weeks	Treatment	Toxin binding normalized by fibre radius	
		^{3}H-STX	^{125}I-BTX
2	control	22.5	11
	denervated	16.8	72.4
	BoTX	17.6	20.3
3	control	64.2	6.9
	denervated	24.5	43.0
	BoTX	44.2	13.4
4	control	72.7	5.59
	denervated	28.8	37.5
	BoTX	43.4	15.7
>7	control	107.8	-
	denervated	64.6	24.2
	BoTX	71.0	21.0

Table 5.2. Toxin binding-site density. Toxin binding sites were normalized for differences in their volume:surface area ratios by multiplying the numbers of binding sites per mg muscle weight by r/2 (r=fibre radius, see Chapter 2). Toxin binding/mg muscle was taken from the experiments of Fig. 5.6 and Fig. 5.10. Fibre radii were from Table 5.1. Extracellular space was not taken into account. Toxin binding site density is compared among control, denervated and BoTX-treated muscles one week after treatment. Rat age is age at death.

controls, in 1 week denervated muscles (Table 5.2). Because the level of ACh receptors is progressively falling with age, the constant proportional increase in the number of ACh receptors at all ages represents a dedifferentiation to progressively less immature states of the muscle membrane. Thus the capacity of the muscle to expand its extracellular ACh receptor complement appears to decrease with muscle maturation.

Neuromuscular blockade with BoTX induced a similar elevation in ^{125}I-BTX binding at all stages of development (Fig. 5.6). As was shown in Figure 5.5 for BoTX-treatment at day 11, BoTX-treatment of neonatal muscles was not equivalent to denervation in increasing ^{125}I-BTX binding. This contrasted with the equivalent effects of denervation and BoTX-treatment in adult rat muscles. When ^{125}I-BTX binding was normalized by muscle fibre radius (Table 5.2), the increase in ^{125}I-BTX binding site densities in BoTX-treated and denervated muscles became more similar with increasing rat age. Therefore, the neuromuscular activity, which can be inhibited by BoTX, appeared to become progressively more important in the regulation of extrajunctional ACh receptors.

5.2.3 Na Channels

5.2.3.1 Development. Numbers of ^{3}H-STX binding sites in neonatal muscle increased from initially low levels to reach adult values by 3-4 weeks (Fig. 5.7; c.f.also Sherman & Catterall, 1982; Lombet et al., 1983). During

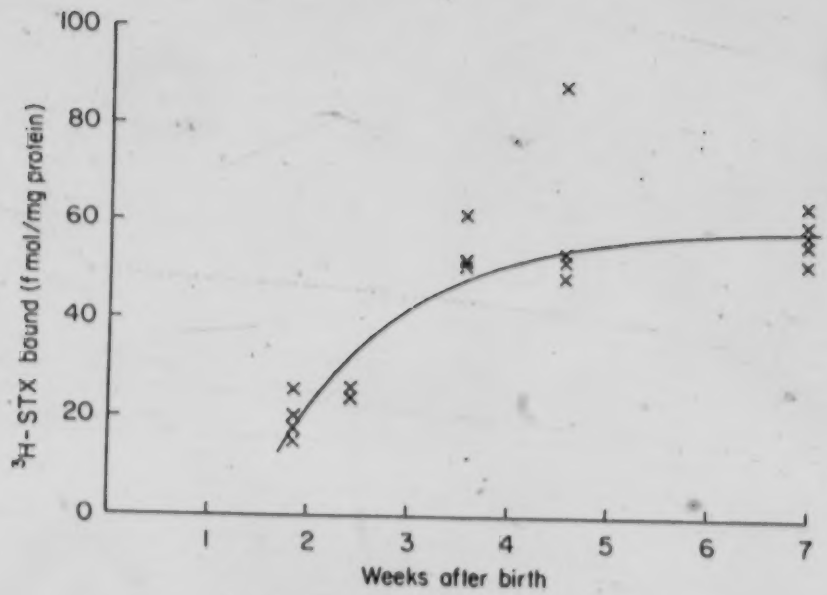


Fig. 5.7. ^{3}H-STX binding to t.s. muscle homogenates as a function of rat age. Binding site numbers are initially low and increase to adult values by 3-4 weeks after birth. Each data point is the calculated value from specific ^{3}H-STX binding to single muscle homogenates (calculated values from direct hyperbolic fits or from Scatchard plots were always in good agreement, Chapter 2).

Fig. 5.8. ^{3}H-STX binding (■) and t.s. muscle protein (▽) as percentages of their adult values with time after birth, compared to the reduction in ^{125}I-BTX binding sites (X) and the increase in t.s. weight (▲) and t.s. weight as a percentage of rat body weight (△) (as shown previously in Fig. 5.4) Muscle ^{3}H-STX site numbers are increasing most rapidly over 1-3 weeks which encompases the time when muscle growth begins to accelerate.

sensitive to the blocking action of TTX (Harris and Marshall, 1973). This suggests that, during development, TTX-insensitive channels may be replaced by or tranformed to the channels measured by high affinity ^{3}H-STX binding. No change in the Kd of the ^{3}H-STX binding was observed during this increase in numbers.

Figure 5.8 shows that incorporation of Na channels is temporally correlated with the muscle growth and preceeded by the decrease in numbers of extrajunctional ACh receptors. The maturation of muscle excitable properties is also well correlated with the onset of adult activity patterns (Navarrete & Vrbova, 1980; 1983; Vrbova et al., 1985).

5.2.3.2 Denervation and BoTX-treatment. Fig. 5.9 compares the specific binding of ^{3}H-STX to normal t.s. muscles of 3 week old rats and to muscles which were denervated by nerve section or treated with BoTX 1 week previously. Both denervation and BoTX-treatment reduced the maximum numbers of ^{3}H-STX binding sites with no change in the Kd. Denervation reduced ^{3}H-STX binding to 36% of control in agreement with earlier reports (Sherman & Catterall, 1982). By comparison BoTX-treatment was somewhat less effective, reducing ^{3}H-STX binding to 51% of control. Thus, not withstanding some recovery of neuromuscular transmission in the neonatal rats by 1 week after BoTX injection, BoTX-blockade reduces the numbers of Na channels which are incorporated into neonatal muscles.

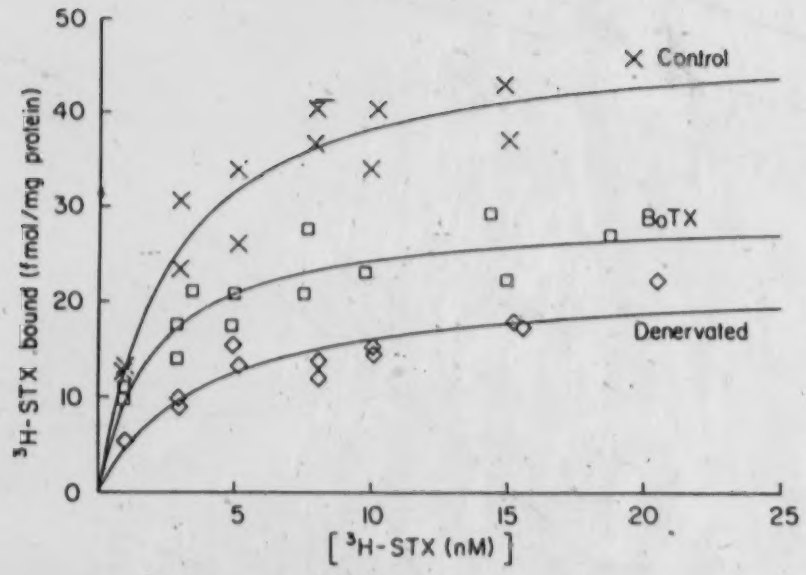


Fig. 5.9. Specific ^{3}H-STX binding to control (X), 1 week denervated (◇) or 1 week BoTX-treated (□) t.s. muscle from 3 week old rats. Both treatments reduce the Bmax of ^{3}H-STX binding without affecting the Kd. The numbers of binding sites in the denervated muscles are lower than in the BoTX-treated t.s. muscles.

The effects of denervation on muscle Na channels are smaller and slower than on muscle ACh receptors. Thus, unlike the results described for ^{125}I-BTX binding, the changes in numbers of ^{3}H-STX binding sites were not significant at 3 days after sciatic nerve section or BoTX treatment: 26±8; 18±3 and 22±5 fmol/mg protein; (X±SE), for control, denervated and BoTX-poisoned muscles, respectively. Thus the question of whether the apparent lesser effect of BoTX-blockade as compared to denervation was due to incomplete neuromuscular blockade by BoTX could not be answered by directly comparing the effects of denervation and BoTX-treatment at the time when neuromuscular transmission was still completely blocked by BoTX. Nevertheless, it is clear that both denervation and BoTX-treatment reduced muscle ^{3}H-STX binding

This effect is larger in neonatal than in adult muscles as shown in Fig. 5.10 where the effects of 1 week of denervation are compared at different stages of development. ^{3}H-STX binding was significantly reduced, relative to age-matched controls when the nerve was sectioned at 2 or 3 but not 1 or 6 weeks of age. By 6 weeks of age, the rat t.s. muscles were mature and showed a response to denervation similar to adult rat muscle (see Chapter 3; Rogart & Ritchie, 1977; Hansen-Bay & Strichartz, 1980; Bambrick & Gordon, 1986b).

As was shown for one experiment in Fig. 5.9, BoTX-paralysis reduced ^{3}H-STX binding in 3 week old rats.

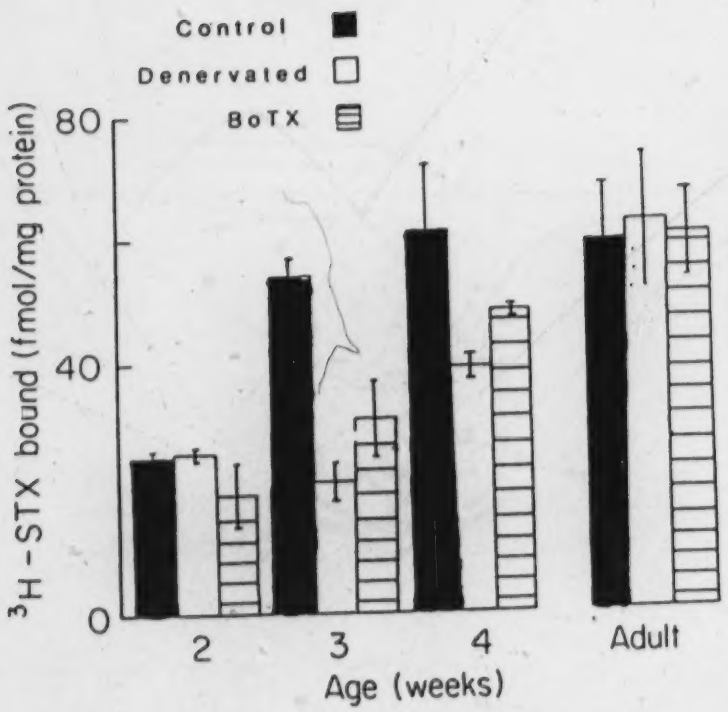


Fig. 5.10. ^{3}H-STX binding to control, 1 week denervated or 1 week BoTX-treated t.s. muscles from rats 2-7+ weeks of age (mean ±SE, n=4-12 muscles).

Similarly to denervation, BoTX-treatment did not change ^{3}H-STX binding in muscles treated at age 1 or 6 weeks. The decrease in muscle ^{3}H-STX binding in 3 week old rats was not significantly different for BoTX-treated and denervated muscles, which suggests that blockade of neuromuscular activity may mimic the effects of denervation in decreasing Na channel incorporation in neonatal as in adult muscle membranes (Chapter 3). Possibly, the differences seen between the two treatments at other ages were due to the recovery of some neuromuscular transmission in juvenile rats. To examine this possibility, the reduction in ^{3}H-STX binding 1 week after denervation or BoTX-treatment was plotted against muscle weight (normalized by rat body weight to compensate for systemic effects of the toxin)(Fig. 5.11). The linear regression line calculated for the BoTX-treated muscle data (RO=0.88; significant at $p<0.01$) passes through the mean for the age-matched 1 week denervated muscles. Since muscles atrophy to a similar extent after complete blockade of neuromuscular transmission with BoTX or after nerve section in adult rats (Chapter, 3); this implies that if complete BoTX blockade is as effective as denervation in reducing t.s. muscle weight, it would also be as effective in decreasing ^{3}H-STX binding.

These results suggest that the shortfall of BoTX-induced changes in Na channels, relative to denervation, was due to the incompleteness of the blockade rather than

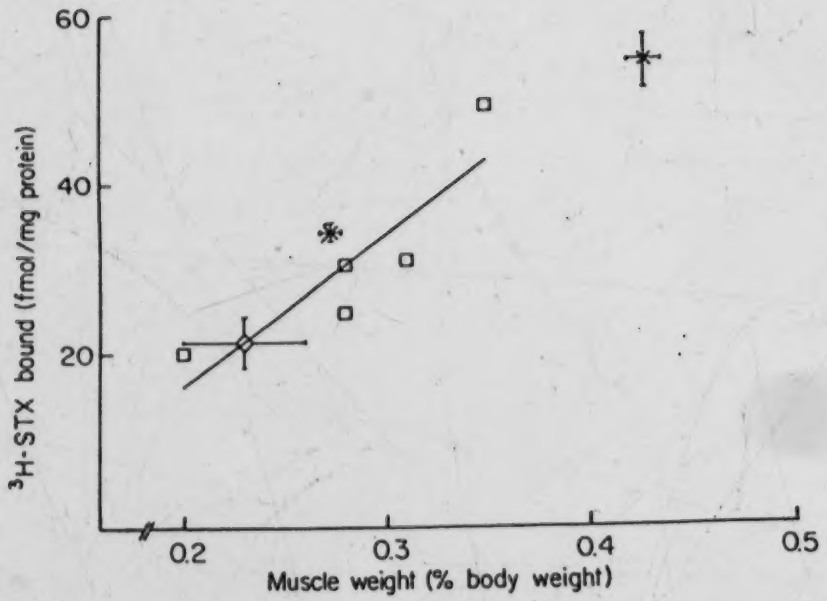


Fig. 5.11. ^{3}H-STX binding as a function of muscle weight (expressed as a percentage of rat weight) in t.s. muscles from 3 week old neonatal rats BoTX injected 1 week previously (□). ^{3}H-STX binding was well correlated with t.s. weight (RO=0.88). Mean±SE of binding sites and muscle weight for comparable denervated (◇) as well as control, untreated 3 week (X, upper symbol) and 2 week (X, lower symbol) t.s. muscles.

to a real difference in the effects of denervation and inactivity. However, the differences in ^{3}H-STX binding site densities in BoTX-treated and denervated muscles cannot be fully accounted for by recovery of neuromuscular transmission in the BoTX-treated rats. At 3 days after BoTX injection, when neuromuscular blockade was complete, neonatal muscles were less atrophic that muscles denervated for the same period. When ^{3}H-STX binding sites are normalized by fibre radius, to control for the lesser atrophy of BoTX-treated muscles (Table 5.2), the decrease in ^{3}H-STX binding site density is up to two-fold greater in denervated than in paralysed muscles.

5.3 DISCUSSION

A number of aspects of the neural control of muscle membrane proteins were demonstrated in this study. 1) During development there is a decrease in extrajunctional ACh receptor density which precedes an increase in muscle Na channels numbers. 2) The rise in Na channel numbers was correlated with increased muscle growth and muscle activity. 3) These changes are mediated by the nerve with neuromuscular activity playing an important role, since the developmental changes are prevented by denervation and inhibited by neuromuscular blockade with BoTX. 4) The effects of BoTX on muscle growth, ACh receptors or Na channels were not equivalent to the effects of denervation, although the differences were due, in part, to the incomplete long-term blockade of neuromuscular

transmission by BoTX in neonatal rats. 5) The results show that activity may be more important in mediating the regulation of neonatal muscle Na channels than of extrajunctional ACh receptors.

5.3.1 Normal Development.

5.3.1.1 ACh receptors. The greatest decline in the incorporation of extrajunctional ACh receptors, measured as ^{125}I-BTX binding, in neonatal muscles occurs over the first 1-2 weeks after birth (Fig. 5.2, 5.3). This decline in muscle ACh receptors with postnatal age is consistent with a number of studies in which different measures of ACh receptor density were employed (Diamond & Miledi, 1962; Devreotes & Fambrough, 1975; Gordon & Vrbova, 1975; Vrbova et al., 1978).

The decline in extrajunctional ACh receptors occurs concurrently with accumulation of receptors at the neuromuscular junction and a change in the properties of the junction receptor. The new junctional receptors become both immunologically (Hall et al., 1985; Schuetze, Vicini & Hall, 1985) and electrophysiologically (Sakmann & Brenner, 1978) distinguishable from neonatal endplate receptors. The long channel open times of the embryonic muscle ACh receptor change to the shorter mean open time of adult receptors during the first week of postnatal life at the same time that extrajunctional ACh receptor density is falling. The isoelectric point (Brockes & Hall, 1975) and degradation rate (Steinbach et al., 1979) of adult and

neonatal junctional receptors do not differ. Immature endplate receptors thus seem to be intermediate between the receptors which appear in the absence of innervation and fully mature junctional ACh receptors.

These findings indicate that some of the characteristics as well as the numbers and location of ACh receptors are regulated by the motoneuron.

5.3.1.2 Na channels. Na channels numbers, measured as ^{3}H-STX binding, increased from very low levels at birth to reach their adult levels by 3 weeks of age (5.7), in agreement with other studies (Sherman & Catterall, 1982; Lombet et al., 1983). A later decline in Na channels 2 months after birth, which was assayed using a tritiated TTX-derivative (Lombet et al., 1983), was not substantiated with ^{3}H-STX as the radioligand for the Na channel in our or other laboratories (Sherman & Catterall, 1982)

The high affinity site of the Na channel for ^{3}H-STX does not change during neonatal development with Kd remaining constant at adult values throughout development (see also Sherman & Catterall, 1982; Lombet et al., 1983). However the TTX-sensitivity of the voltage-dependent Na current of the action potential increases sharply over the first 2-3 weeks after birth (Harris & Marshall, 1973). This correlates with the appearance of high affinity ^{3}H-STX binding sites (Fig. 5.8) and suggests that the appearance of high affinity binding sites for ^{3}H-STX and

of TTX-sensitivity are co-regulated, with channels having low affinities for the neurotoxins being replaced by or transformed to channels with high affinities for the toxin.

Consistent with a role for the nerve in maintaining high affinity toxin binding sites, low affinity sites reappear after denervation of adult muscle (Rogart & Regan, 1985); TTX-resistant Na channels are also present in aneural skeletal muscle cultures from rat (Frelin et al., 1983) and chicken (Baumgold et al., 1983a;b; Strichartz et al., 1983) and Na channels in rat skeletal muscle in vitro do not develop TTX-sensitivity in the absence of innervation (Frelin et al., 1984). Thus, as is the case for the ACh receptor, innervation regulates numbers and properties of the muscle Na channel.

5.3.1.3 Correlation of muscle development with muscle activity. During postnatal development, acceleration in muscle growth coincides with the time when the eyes open and neonatal rats begin to move independently as the pattern of neuromuscular activity changes to establish adult patterns 2-3 weeks after birth (Navarrete & Vrbova, 1980; 1983; Vrbova et al., 1985). This also coincides with the time of elimination of polyneuronal innervation (section 5.3.1.3).

Numbers of extrajunctional ACh receptors begin to decline after muscle innervation in utero. The persuasive evidence for a role of neuromuscular activity in reducing

the synthesis and incorporation of extrajunctional ACh receptors (see below) makes it plausible that this decrease could be mediated by the low levels of prenatal neuromuscular activity. This decline is temporarily halted across the perinatal period possibly as a result of a transient decrease in muscle activity due to uncoupling of gap junctions between muscle fibres at this time (Schmalbruch, 1982). The rapid postnatal decline in extrajunctional ACh receptors, which precedes the spurt in muscle growth and activity, suggested that synthesis and incorporation of ACh receptors during postnatal muscle development may not be as closely regulated by muscle activity as are Na channels and muscle growth.

The finding that increased expression of Na channels occurred over the time of the accelerated muscle growth and neuromuscular activity indicated that membrane excitability, in contrast to chemosensitivity, is very sensitive to muscle activity during development. Another finding suggesting that neuromuscular activity is important in the regulation of Na channel incorporation is that ^{3}H-STX binding sites appear earlier in the postnatal development of the more active diaphragm than in the hindlimb muscles (Sherman & Catterall, 1982). Diaphragm muscle is more active earlier in development than hindlimb muscle due to a rostral-caudal gradient of muscle maturation and the obligatory use of respiratory muscles immediately after birth, while the weight bearing muscles

of the hindlimb are used later.

5.3.2 Effects of nerve section or BoTX-treatment on developing muscle.

Interruption of nerve-muscle communication, during the differentiation of skeletal muscle chemosensitivity and electrical excitability, by nerve section or by blockade of ACh release with BoTX halted or reversed this differentiation.

5.3.2.1 ACh receptors. Denervation of neonatal muscle dramatically increased the incorporation of extrajunctional ACh receptors thereby reversing the normal developmental differentiation. Similarly, denervation has been found to prevent the electrical differentiation of the ACh receptor channel from the neonatal form with long mean channel open time, to the adult channel with short mean channel open time. (Schuetze & Vicini, 1984).

The denervation of neonatal muscles resulted in a dedifferentiation of the muscle membrane to an embryonic state as the denervated neonatal muscle expresses levels of ^{125}I-BTX binding higher than those normally found on postnatal muscle fibres (Table 5.2) and such high densities of ACh receptors have been reported previously for embryonic fibres shortly after innervation (Bevan & Steinbach, 1977).

BoTX-treatment also increased ACh receptor numbers but its effects were not equivalent to denervation. The results show that only part of the neuronal regulation of

neonatal muscle ACh receptors is regulated by BoTX-blockable neuromuscular activity, in contrast to the regulation of the receptor in adult muscle (Chapter 3). The difference was not due to the incomplete blockade of neuromuscular transmission achieved by BoTX over periods longer than 3-4 days. Normalization of ^{125}I-BTX binding sites by muscle fibre radius allowed a comparison of binding site density in denervated and BoTX-treated muscles (Table 5.2) and showed that BoTX became more effective in increasing extrajunctional ACh receptor density with increasing rat age. Therefore, some factor is important for the regulation of the ACh receptor in immature, but not mature muscle, and this factor becomes less important as the muscle develops.

5.3.2.2 Na channels. The ^{3}H-STX binding site densities which were calculated using the muscle fibre radii are only approximations since incorporation of Na channels into the t-tubular membrane was not taken into consideration (Jaimovich et al., 1976; 1983; 1985). In adult muscle, these t-tubular Na channels form a significant proportion of the total (cf. Chapter 3). The estimates in Table 5.2 have not been further refined since no information is available, to date, on the development of Na channel density in neonatal muscle t-tubules. Nevertheless, it is clear that the dependence of Na channel expression on innervation declined progressively with age during development.

The finding that denervation of neonatal muscle within the first week of life was without measurable effect on muscle ^{3}H-STX binding sites may be due to a stimulatory effect of denervation on muscle Na channel synthesis early in _in vivo_ development (Sherman & Catterall, 1982). This suggests that early neuromuscular activity may actually inhibit Na channel synthesis at this stage. Consistent with this, the numbers of ^{3}H-STX binding sites on myotubes _in vitro_ increased in the presence of TTX (Sherman & Catterall, 1984).

Later effects of denervation in inhibiting Na channel synthesis may reflect a dedifferentiation of the muscle membrane. Denervation of neonatal muscles reduces numbers of Na channels to 50% of the adult maximum, as also shown previously by Sherman & Catterall (1982), which implies that half of the Na channels are neuronally regulated while half are incorporated regardless of the innervation of the muscle. Consistent with this, aneural muscle cells in culture incorporate Na channels (Catterall, 1980; Frelin et al., 1981; Strichartz et al., 1983). Slightly more than half the Na channels may be neuronally regulated because concurrent atrophy of muscle fibres, which influences calculations of Na channel density, was not accounted for in the normalization of ^{3}H-STX binding sites by muscle weight in Sherman & Catterall's study (1982). Denervation becomes less effective with age (Fig. 5.10) soon after the time when the plateau values of ^{3}H-STX

binding sites are incorporated. This suggests that the expression of the Na channel becomes less plastic to neuronal influence as the muscle membrane matures.

At least part of the neuronal regulation of muscle Na channels is mediated by neuromuscular activity since BoTX-treatment decreased Na channel numbers in neonatal muscle, although consistently less than did denervation. As suggested by the closer correlation between muscular activity and incorporation of ^{3}H-STX binding during development than with the decline in extrajunctional ACh receptors (5.3.1.4), BoTX more closely mimicked denervation in decreasing Na channels than in increasing ACh receptors. The nature of the neuronal influence is considered further in section 5.3.4.

5.3.2.3 Muscle atrophy. The developmental increases in muscle mass and protein content are not completely under neuronal control, since growth continued despite denervation. Nevertheless, denervation and, to a lesser extent, neuromuscular blockade with BoTX reduced muscle weight gain during neonatal development argueing that the motor nerve, and particularly neuromuscular activity, has an important regulatory role. By 3-4 weeks after birth, neuronal regulation becomes more prominent, as shown by the finding that 1 week of denervation completely halted muscle growth and BoTX-treatment severely retards it (Table 5.1). After the initial growth, muscle weight and protein content become more exclusively regulated by

innervation and this regulation is, at least partially, mediated by neuromuscular activity.

5.3.4 Mechanisms of neuronal regulation of muscle

5.3.4.1 Muscular activity. The temporal correlation of muscle growth and Na channel incorporation with the development of adult amounts and patterns of muscle activity (Navarrte & Vrbova, 1980; 1983; Vrbova et al., 1985) during development in the rat hindlimb provides indirect evidence that neuromuscular activity is a mediator of the neuronal regulation of muscle properties . More direct evidence was provided by experiments in which denervation and neuromuscular blockade with BoTX-treatment altered the normal differentiation of muscle chemosensitivity and electrical excitability.

In adult muscle (Chapter 3; Brown et al., 1982), BoTX-treatment mimicked denervation in inducing muscle atrophy, increased numbers of ACh receptors and decreased numbers of high affinity Na channel toxin binding sites. In neonates, denervation was always more effective than BoTX-blockade. Muscle atrophy after BoTX-treatment was directly correlated with inhibition of Na channel expression but not with an increased expression of ACh receptors. The effect of BoTX on ^{125}I-BTX binding also less closely mimicked denervation than did its effects on Na channels and muscle weight. Although neuromuscular blockade affected all the measured muscle properties, membrane excitability was relatively more dependent on muscle

activity than chemosensitivity.

The differences between denervated and BoTX-treated neonatal muscles implies the existence of a mediator of nerve-muscle interactions other than activity. Alternatively, it could be postulated that BoTX never completely blocked neuromuscular transmission in neonatal rats and that the muscles were never completely inactive. While it is certainly true that transmission recovered to some extent by 1 week, hindlimb muscles in neonatal rats 2-3 days after BoTX injection were paralysed and were still less atrophic, with lower levels of ^{125}I-BTX binding sites than comparable denervated muscles. Other possible factors contributing to the differences observed between denervated and BoTX-treated neonatal muscles include the presence of spontaneously released ACh or other trophic substances at the BoTX-poisoned nerves terminals and the presence of products of nerve degeneration at denervated muscles.

5.3.4.2 Acetylcholine. BoTX blocks all evoked but only a portion of the non-evoked release of ACh from the nerve terminal (Dolezal et al., 1983; Gunderson & Jenden, 1983; Stanley & Drachman, 1983). Since ACh itself has been proposed as a trophic factor (Thesleff, 1960; Drachman, 1967; Mathers & Thesleff, 1978) it is possible that the remaining ACh present at the BoTX-treated neuromuscular junctions in neonatal rats was sufficient to prevent the full expression of denervation-like effects in these

muscles. It is difficult to estimate how much ACh might be present at the BoTX-blocked neonatal junctions because there has been some controversy as to the proportion of non-evoked ACh release blocked by BoTX in adult nerve-muscle preparations (Dolezal et al., 1983; Gunderson & Jenden, 1983; Stanley & Drachman, 1983) and no quantitative studies on BoTX blockade of ACh release in neonatal muscles have been reported. If ACh is an important trophic factor in adult rats, the ACh release blocked by BoTX in the adult rats is evidently sufficient for its effects to be equivalent to those of denervation (Chapter 3). However, there are several reasons why neonatal muscles may be more sensitive than adult muscles to the presence of spontaneously released ACh.

Neonatal rat hindlimb muscles are polyneuronally innervated up to 3 weeks after birth (Redfern, 1970; Bennett & Pettigrew, 1974; O'Brien et al., 1978) and therefore more ACh may be released onto the neonatal muscles. AChE density is not fully developed at the immature junction and this permits unhydrolysed ACh to remain in the cleft longer than at the synaptic cleft of adult muscles (Tucek, 1972; Bennett & Pettigrew, 1974). This ACh could exert a trophic effect by interacting with some, as yet undiscovered, site on the muscle membrane or by some event consequent on its interaction with the nicotinic ACh receptor. The binding of ACh to its receptor in skeletal muscle results in a depolarization of the

muscle membrane. Membrane depolarization has been previously reported to be capable of exerting a trophic influence on muscle as shown by its supression of extrajunctional muscle membrane ACh receptors (Gruener et al., 1974). This effect would be augmented in immature muscle because of the greater number of ACh receptors, the longer channel open times of the ACh receptors present (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980) and their higher input resistence, consequent to the smaller fibre diameter (Katz & Thesleff, 1957). The lesser length of the neonatal muscles would also result in a larger proportion of the muscle being activated by a sub-threshold depolarization of a given amplitude than would be affected in a longer fibre. Consequently the extent of the depolarization resulting from the presence of a given concentration of ACh could be larger and more wide-spread in immature as compared to adult muscles.

As discussed below, Ca has been implicated as an important second messenger in the regulation of muscle ACh receptors and Na channels. Ca may cross the muscle membrane when it is depolarized and is released from intracellular stores. Immature muscles may be less able to remove Ca from the cytoplasm than adult muscles (Zubrzycka-Gaarn & Sarzala, 1980) and this Ca may contribute to the augmentation of the trophic effect of ACh in the neonatal muscles. Therefore a trophic role for spontaneously released ACh is consistent both with the

observed difference in the efficacy of BoTX in modulating muscle properties in mature versus developing rats and with the lesser effect of BoTX, as compared to denervation, in the latter animals.

5.3.4.3 Other possible trophic factors. Another possible explanation of the difference between the effects of BoTX and nerve section on developing muscle is that the blocked nerve releases some non-ACh trophic factor. Possibly the trophic substance is no longer released from mature motoneurons or, alternatively, mature muscles may have lost receptors for the trophic agent. Several papers have reported the induction of aggregation of ACh receptors and AChE on muscle membranes following the addition of nerve extracts or media from cultures of neurons (Schaffner & Daniels, 1982). These two molecules also cluster at the developing neuromuscular junction and the aggregating factors have been proposed as trophic substances. The finding that ACh receptors also cluster in response to the presence of degenerating nerve terminals (Vrbova, 1967; Jones & Vrbova, 1974; Jones & Vyskocil, 1975) or injury (Katz & Miledi, 1964; Jones and Vrbova, 1974) would indicate that clustering alone is not necessarily a good model of the presence of a specifically trophic factor. In addition, although the action of these factors is consistent with the observed effect of the nerve at the neuromuscular junction, it has not been demonstrated that such factors do decrease extrajunctional ACh receptor

density, induce muscle growth and the maturation of Na channels. The possibility that some, as yet undiscovered, trophic substance is important in regulating muscle development cannot be excluded.

The observation that products of nerve degeneration can increase numbers of muscle ACh receptors (see above) may explain the results of Brown et al. (1982) where the ACh sensitivity of BoTX-treated mouse muscles became equivalent to that of denervated muscles only after a delay of up to 9 days. Such a delay was not found for adult rats (Chapter 3) One difference between the two studies was the relative youth of the mice used by Brown et al. (1982). If immature muscles are more sensitive to the presence of nerve breakdown products than adult muscles, then this would explain the difference between the 2 studies. Possibly BoTX-blockade would induce changes in the muscle equivalent to denervation if the blockade was maintained for a longer period of time. Due to the rapid recovery neuromuscular transmission in BoTX-blocked neonatal muscles (Chapter 8), however, this hypothesis could not be tested.

CHAPTER SIX: REGULATION OF MUSCLE ACETYLCHOLINE RECEPTORS AND SODIUM CHANNELS: A DISCUSSION

6.1 ACh receptor

The skeletal muscle membrane contains two populations of ACh receptors, one located in the junctional and the other in the extrajunctional membrane. Innervation appears to increase ACh receptor numbers at the neuromuscular junction and decrease them elsewhere in the membrane. Evidence presented here and in other reports (see Chapter 1) demonstrates that the extrajunctional ACh receptor density is maintained at very low levels in adult muscle by neuromuscular activity. Both evoked and non-evoked release of ACh would appear to be important in this regulation since quiescent cat gastrocnemius muscles developed increased ^{125}I-BTX binding but were less similar to denervated muscles than were BoTX-treated rat t.s. muscles - in which some of the non-evoked release of ACh was blocked (Dolezal et al., 1983; Gunderson & Jenden, 1983; Stanley & Drachman, 1983).

In neonatal rats the effect of BoTX was not equivalent to that of denervation in increasing muscle ACh receptor numbers, but BoTX-blockable neuromuscular activity was shown to be a factor in the developmental decrease in extrajunctional ACh receptor density. In Chapter 5, arguments were presented to the effect that non-evoked release of ACh would have a larger depolarizing effect on neonatal than on adult muscle fibres and it was suggested,

therefore, that the non-evoked ACh not blocked by BoTX was an important trophic factor for developing muscles. The non-evoked release of ACh could reduce muscle chemosensitivity via a sub-mechanical threshold depolarization of the membrane (Gruener et al., 1974).

This does not exclude a possible involvement of other nerve-released factors in the regulation of muscle ACh receptors. Possibly BoTX interferes with release of some trophic agent other than ACh and this is why BoTX mimics denervation in adult rats. However, the changes seen in the quiescent cat muscles, where a trophic substance could still be released, argue against a large role for such a substance in regulating extrajunctional ACh receptor density. Products of nerve degeneration have been reported to increase extrajunctional ACh receptor numbers and may be important in the early phase of denervation supersensitivity. In the experiments reported here, such products did not appear to be playing a large role in denervated adult rat muscles but possibly immature muscles are more affected by the degeneration products and this may also have contributed to the observed difference between the magnitude of the increase in ACh receptor numbers induced by denervation and by BoTX-paralysis of immature muscle.

ACh receptor numbers at the neuromuscular endplate increase following innervation. This may be due to a stabilization of the receptors in the membrane by other

postsynaptic specializations. It has been suggested that the developmental change in ACh receptor mean channel open time is a post-translational event, although there is also some evidence that the ACh receptor of immature muscle has a subunit composition which differs from that of the adult receptor (Mishina, Takai, Imoto, Noda, Takahashi, Numa, Methfessel & Sakmann, 1986). Many adhesive macromolecules accumulate at the neuromuscular junction and may be important in the stabilization of high ACh receptor densities (Bayne et al., 1984; Covault & Sanes, 1986; Sanes, Schachner & Covault, 1986). In this light, it may be significant that the levels of one such macromolecule, the nerve-cell adhesion molecule (N-CAM), are elevated in denervated muscle (Covault, Merlie, Goridis & Sanes, 1986). Possibly this changes the extrajunctional membrane so that it will support higher extrajunctional ACh receptor accumulations.

The control of extrajunctional ACh receptor synthesis by neuromuscular transmission may be initially mediated by some change in the muscle membrane which then feeds back onto receptor protein synthesis, or by a direct action of neuromuscular activity on gene expression. mRNA levels for ACh receptor rise after denervation (Merlie, Isenberg, Russell & Sanes, 1984) so that, whatever the mechanism, denervation does affect muscle events at the nuclear level. Activity may restrict ACh receptor synthesis by restricting the transcription of ACh receptor DNA to

nuclei in the peri-endplate region (Sanes & Merlie, 1984) and this restriction may disappear after denervation. The neuronal control may be mediated by a Ca dependent step; experiments have shown that the effect of inactivity, which increases ACh receptor numbers on myotubes in culture, are reversed in the presence of a Ca ionophore. Other second messengers, such as cAMP or cGMP, did not seem to be important for the regulation of ACh receptor numbers in vitro (McManaman, Blosser & Appel, 1982). Altered Ca levels could affect intracellular events by activating Ca-dependent kinases and thus inducing the phosphorylation of other proteins.

6.2 Na channels

The distribution of Na channels in the sarcolemma has not been as extensively studied as the distribution of ACh receptors. There is a gradient of channel density which peaks at the endplate region (Beam et al., 1985) and may, as seems to be the case for the elevated Na channel density at the axon hillock of neurons (Catterall, 1981) be important for increasing the safety factor for initiation of an action potential. No evidence is available as to how this gradient is established during development although it may persist after denervation (Caldwell & Milton, 1986).

The temporal correlation between the development of ^{3}H-STX sensitive Na channels and muscle activity suggested that activity is important in the regulation of the Na

channel. This is supported by the effects of denervation and BoTX blockade in depressing channel density in mature and developing muscle. Na channel density does not appear to be as plastic to neuronal influence as are ACh receptor numbers. Channel numbers in skeletal muscle are still low at birth while ACh receptor density begins to decline immediately after innervation in utero. In addition, a certain fraction of the Na channel population is incorporated into the membrane in the absence of continuing innervation of the muscle (cf also Sherman & Catterall, 1982) during development. Also, denervation of adult muscle does not result in the conversion of the entire muscle Na channel population to the immature channel form (cf Pappone, 1980). Thus, unlike extrajunctional ACh receptors, Na channels consist of a neuron-dependent and a neuron-independent population.

During muscle development, the later appearance of Na channels and the greater effect of BoTX-blockade on their numbers, relative the ACh receptor levels, suggest that Na channels are regulated by neuromuscular activity but are less sensitive than extrajunctional ACh receptors to low activity levels. Possibly channel numbers are only regulated by supra-threshold depolarizations of the muscle membrane. As was discussed for ACh receptors (6.1) products of nerve degeneration may play a role in decreasing Na channel numbers in denervated immature muscle and this may again contribute to the difference

between the effects of denervation and BoTX-blockade in young rats. Similarly to the regulation of the ACh receptor, the regulation of Na channels in vitro may involve a Ca-dependent step (Sherman & Catterall, 1984) and the second messenger cAMP has been shown to exert an opposite effect to Ca on Na channels in cultured muscle (Sherman, Chrivia & Catterall, 1985).

The differentiation of the Na channel with the development of TTX-sensitivity is apparently dependent upon innervation, since rat muscle cells in culture don't appear to develop TTX-sensitive action potentials. Continuing innervation is neccessary for the appearance and maintenance of TTX-sensitivity in the whole muscle Na channel population in vivo since denervation of adult muscle results in the reversion of approximately 30% of the Na channels to a TTX-insensitive state (Pappone, 1980). Future experiments on the neuronal control of the Na channel using ^{125}I-ScTX binding to whole muscle or radiolabelled batrachotoxin or veratridine binding to muscle homogenates from normal, denervated and paralyzed muscles from adult and developing rats would contribute to our understanding of the neuronal control of the Na channel as a molecular entity.

6.3 Conclusion

Evidence has been presented in this thesis consistent with a regulation of muscle ACh receptors and Na channels by neuromuscular activity in developing and mature rat

muscle. Both evoked and non-evoked release of ACh are important in this regulation and are suggested to be acting via the depolarization of the muscle membrane. Products of nerve terminal degeneration may affect ACh receptor and Na channel numbers shortly after denervation and immature muscle may be more sensitive to such products. Extrajunctional ACh receptors and Na channels are not co-regulated during development or after interruption of neuromuscular communication but this appears to be the result of the greater plasticity of ACh receptor numbers to ACh rather than of a different mode of neuronal regulation of the two proteins.

CHAPTER SEVEN: INCORPORATION OF SODIUM CHANNELS IN DEVELOPING MAMMALIAN BRAIN

7.1 Introduction

The ability to conduct regenerative action potentials is characteristic of excitable cells. In adult mammalian neurons, the inward current responsible for the rising phase of the action potential is carried primarily by Na ions through a voltage-sensitive Na channel. This channel is electrophysiologically and pharmacologically similar to the Na channel in adult muscle (Lawrence & Catterall, 1981a,b).

The development of the Na channel as a molecular entity, as well as developmental changes in the number of channels can be studied with radioligand binding because radiolabelled ligands are available which bind to different sites on the channel (Catterall, 1977; Stricharz, 1981). As described in Chapter I, the guanidinium ion toxins, TTX and STX, bind to a site on the extracellular face of the channel and inhibit Na passage through the channel pore (Catterall & Morrow, 1978; Kao & Walker, 1982; Strichartz, 1981; Rubinson, 1982). The scorpion toxins bind to a site on the intracellular face of the channel protein, at or near the inactivation gate, and inhibit Na channel inactivation. The binding of ScTX to its site on the channel is dependent on the existence of the membrane potential (Narahashi et al., 1972; Catterall, 1976; 1977; 1979; Ovchinnikov & Grishin, 1982;

Meves et al; 1984).

An earlier study of the Na channel by Baumgold et al. (1983a) showed that the appearance of ^{125}I-ScTX binding sites preceded the appearance of ^{3}H-STX sites in chick muscle developing *in vitro* and that this was correlated with physiological measures of the effects of the two toxins on Na currents in these muscle cells, ie. Na currents could be prolonged by the addition of ScTX at a time during development when the same currents were not inhibitable by STX or TTX (Baumgold et al., 1983b). Because ScTX binding is voltage dependent, significant binding cannot be measured in muscle homogenates and so it is difficult to study the maturation of Na channels in muscle *in vivo* using ligand binding. However, brain synaptosome preparations maintain a potential difference across the membrane and are suitable for use in ^{125}I-ScTX binding experiments.

In this study, the development of the Na channel was studied by comparing the time courses of the appearance of ^{3}H-STX and ^{125}I-ScTX binding to rat brain developing *in vivo*. The results were compared with Baumgold et al. (1983a,b) to test the hypothesis that Na channels in different excitable tissues pass through similar stages during maturation. The time course of Na channel development, with regard to the maturation of the channels and the regulation of channel numbers during development, was compared with reports of concurrent developmental

changes in the brain in order to assess the possible roles for activity, and other cell-cell interactions, especially with the myelinating glial cells, in regulating neuronal Na channels.

7.2 RESULTS

7.2.1 ^{3}H-STX binding sites in developing rat brain.

Significant levels of specific ^{3}H-STX binding can be measured in adult rat brain. Comparison of the specific ^{3}H-STX binding to fetal, neonatal and adult rat brain, shown as hyperbolic curves and Scatchard plots in Fig 7.1, demonstrates that ^{3}H-STX binding increased with age. The Bmax for 19 day *in utero* brain is only 97 fmol/mg protein which increased to 297 at two days after birth,, rising to 750 fmol/mg protein in the adult animal. In contrast, the affinity of the ^{3}H-STX site, as measured by the Kd, did not change with age (Baumgold et al., 1983). For the data in Fig. 7.1 the values for the Kd's were calculated as 0.9, 1.2,and 1.3 nM for binding in the fetal, neonatal and adult rat brains, respectively. The density of ^{3}H-STX sites rises very rapidly over the first three postnatal weeks and then reaches the adult values (Table 7.1). The Kd averaged 1.0 nM for all ages.

7.2.2. Comparison of ^{3}H-STX and ^{125}I-ScTX binding in developing rat brain.

As described in Chapter 2, ^{125}I-ScTX binding, being voltage-dependent, was assayed using rat brain synaptosomes. In this preparation, total binding was well differentiated from the nonspecific allowing good

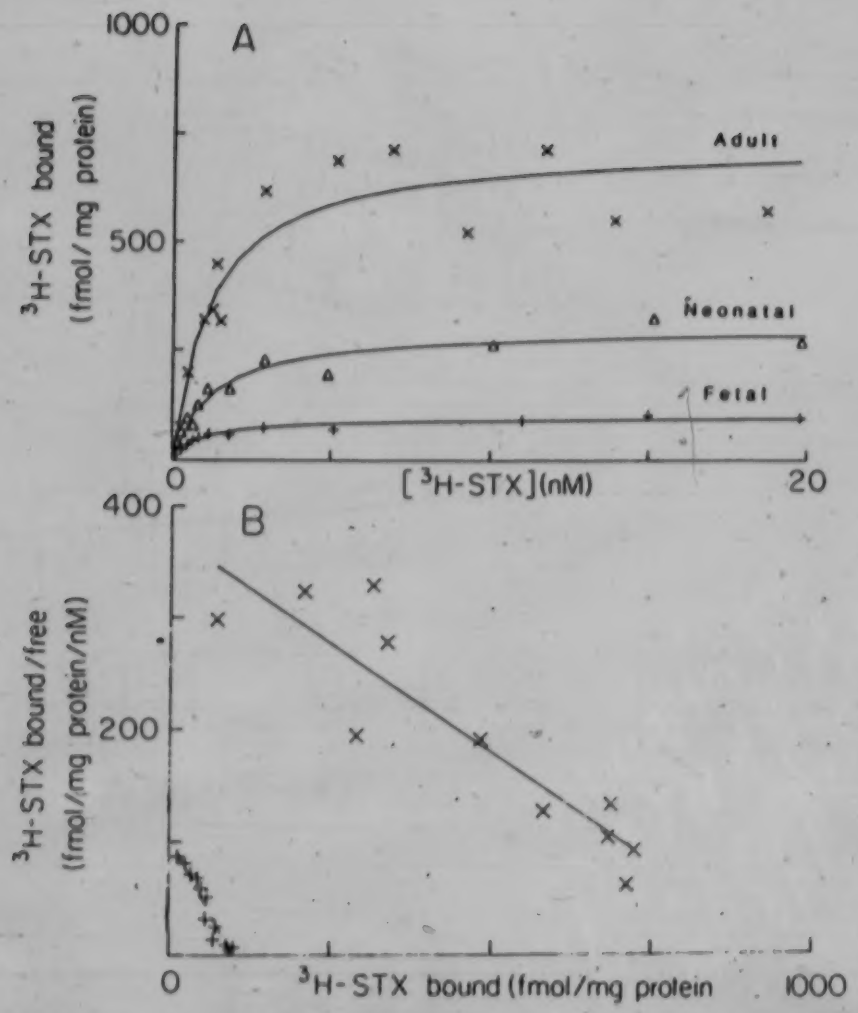


Fig. 7.1. ^{3}H-STX binding to brain homogenate preparations from adult (X), 2-3 day postnatal (Δ) and 19 day in utero (+) rats. A. specific binding fitted with hyperbolics, B. Scatchard plots. Note that the Bmax increases with age while the Kd is unchanged.

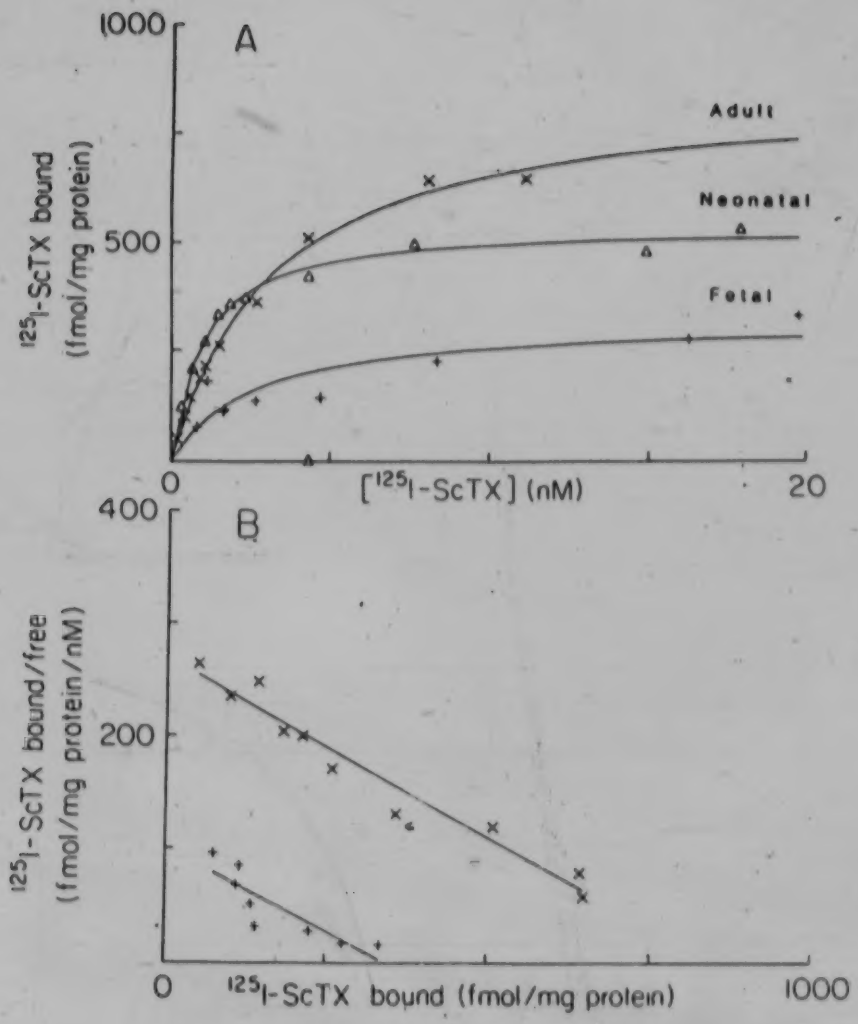


Fig. 7.2. ^{125}I-ScTX binding to synaptosomal preparations from adult (X), 2-3 day postnatal (Δ) and 19 day in utero (+) rat brain A. specific binding fitted with hyperbolic curves, B. Scatchard plots. Note that the Bmax increases with age with no significant change in the Kd.

resolution of the specific binding. Bmax and Kd were again calculated from the directly fitted hyperbolic or from the Scatchard plot.

Similarly to ^{3}H-STX binding, ^{125}I-ScTX binding also increased with age with no change in the Kd (Fig.7.2). However, 2 days before birth, when ^{3}H-STX binding was very low (12% of normal adult values), the numbers of ^{125}I-ScTX sites were nearly 40% of adult numbers rising to 60% by 2-3 days postnatal when ^{3}H-STX sites are only 40% of their adult maximum (Table 7.1). Thus, as was found for chick muscles in vitro (Baumgold et al., 1983a,b), during the in vivo development of the Na channel in rat brain, maximum ^{125}I-ScTX binding was attained before maximum ^{3}H-STX binding.

7.3 DISCUSSION

These results show that Na channels are incorporated progressively with time into developing rat brain during the first month of postnatal life. Specific neurotoxin binding sites on the Na channel were differentially regulated during development, although the affinity for the toxins at their binding sites did not change.

Since ^{125}I-ScTX binding was assayed in synaptosomes and ^{3}H-STX binding in rat brain homogenates, it could be argued that these apparently different time courses actually reflect an earlier appearane of Na channels in synaptosomal membrane relative to whole brain, rather than a differential expression of toxin sites on the Na

Age(days)	% of adult levels of high affinity Na Channel toxin binding sites	
	^{125}I-ScTX	^{3}H-STX
-2	40	12
2-3	60	40
56	100	100

Table 7.1. Developmental increase in ^{125}I-ScTX and ^{3}H-STX binding, relative to their adult (56 day), values as a function of age after birth. Note the earlier appearance of ^{125}I-ScTX binding.

channel. However, a more recent study used a single synaptosomal preparation for assays of both ^{125}I-ScTX and ^{3}H-STX binding and again reported that the rise in ^{125}I-ScTX binding in developing rat brain preceeded the increase in ^{3}H-STX binding (Baumgold, 1985). Therefore, these results are not an artifact of the preparations used in the measurement of toxin binding but appear to genuinely indicate that the Na channel exists in mature and immature states during the development of rat brain.

Before reaching this conclusion, another possibility must be considered. Recently, Na channels have been found in several non-neuronal cell types. These include rabbit cultured Schwann cells (Chiu et al., 1984), primary cultures of astrocytes (Bowman, Kimelberg, Frangakis, Berwald-Netter & Edwards, 1984) and other CNS glial cells (Reiser et al., 1983). It has been suggested that these Na channels are inactive because the resting potential in these cells is much smaller than in nerve cells (Chiu et al., 1984). Therefore, if there was some glial contamination of the synaptosomal preparation, it would not contribute to the ^{125}I-ScTX binding as binding of this toxin is voltage-dependent. The appearance of Na channels in neuronal cells would, therefore, cause a simultaneous increase in ^{125}I-ScTX binding and ^{3}H-STX binding whereas the appearance of Na channels in glial cells would only be measurable as an increase in ^{3}H-STX binding. Since most glial cells are formed postnally (Berwald-Netter et al.,

1982) the delay in reaching maximal levels of ^{3}H-STX binding, relative to ^{125}I-ScTX binding, in brain could be due to the different time courses of appearance of neuronal and glial cells.

This explaination of the apparent differential regulation of the two Na channel ligand binding sites is unlikely for several reasons. Firstly, Na channels which are activated by ScTX, but not affected by nM concentrations of STX have been reported in cultures of single cell types (Lawrence & Catterall, 1981a,b; Baumgold et al., 1983a,b), therefore, there is a precedent for suggesting a differential regulation of the 2 toxin sites. Secondly, although non-neuronal cells in vitro may express voltage-activated Na channels, the available evidence suggests that glial cells in the mammalian CNS in vivo may not bind STX or TTX (Buonnano & Villegas, 1983; Politis, Pellegrino, Oaklander & Ritchie, 1983).

Although numbers of ^{3}H-STX and ^{125}I-ScTX binding sites measured in this study are not directly comparable, since measures of toxin binding were carried out on different rat brain preparations, normalizing each set of binding site numbers by its adult plateau value allowed a comparison of the relative maturity of the 2 sites on the Na channel at different ages (Table 7.1). Two days after birth, only 30% of the Na channels appear to express high affinity binding sites for STX with this value rising to 100%, 50 days after birth.

Therefore it appears that two forms of the Na channel exist, one of which appears early in development and does not have a high affinity ^{3}H-STX binding site. This earlier form of the channel then gradually gives way to the adult, ^{3}H-STX binding, form of the channel. Thus, there are two aspects to the regulation of the Na channel during development. One is the increase in channel numbers with age and the second is the maturation of the Na channel with the appearance of adult levels of ^{3}H-STX binding. In the following sections, possible roles for neuronal activity and interactions with other cell types in the regulation of the Na channel will be considered.

Using ^{125}I-ScTX binding as a measure of the appearance of Na channels in developing rat brain, most of the channels appeared after 19 days in utero. The majority of the neurons in the rat cortex (Berry & Rodgers, 1965), hippocampus and cerebellum (Altman & Anderson, 1972) originate before day 19 in utero with only some layer I and II cortical cells and cerebellar interneurons born between day 19 in utero and day 56 postnatal, the times when ^{125}I-ScTX binding site numbers were assayed. The fact that most of the neurons are born before the increase in ^{125}I-ScTX sites would seem to preclude the possibility that the increase is due simply to the appearance of more Na channel containing cells.

The hypothesis that activity is important for the regulation of Na channels during development in brain, as

it is for Na channel development in muscles (Chapter 5), was examined by comparing the time of maximal synapse formation with the time course of the appearance of ^{125}I-ScTX binding sites. Connections between neurons continue to be formed long after neurogenesis (Altman, 1967) and most synapses are formed after birth. The number of synapses per unit area, as viewed with an electron microscope, increased three-fold from five hours to six days postnatal (Kristt & Molliver, 1976) but the maximal increase of synapses in cortex occurred some time later, between day 12 and 20 postnatal (Aghajanian & Bloom, 1967), at the time of maximal increase in cortical axon density (Eayers & Goodhead, 1959). The time of maximal synaptogenesis does not, therefore, correlate well with the time of maximal ^{125}I-ScTX binding site incorporation. Synaptogenesis occurs rather later than the initial incorporation of Na channels and thus there is no clear evidence for an inducement of the appearance of Na channels by neuronal activity.

In myelinated nerves, Na channels are localized to the nodal membrane and are not found along the inter-nodal axonal membrane (cf. Ritchie & Rogart, 1977; Waxman & Foster, 1980; Meirie et al., 1984). The time course of myelination in mammalian brain was correlated with the appearance of Na channels in order to examine the possibility that myelination could be a signal for the incorporation of Na channels by the nerve. Myelination in

rat brains occurs primarily after birth. Some sheaths are visible by two days after birth (Jacobson, 1963) but most cells are still unmyelinated by day 10 (Sherwood & Timiras, 1970). Most myelination occurs between days 15 and 21 with adult levels attained by day 39 (Jacobson, 1963; Sherwood & Timiras, 1970), which is very similar to the time course reported for myelination in mouse brain (Schonback, Hu & Friede, 1968). As this is well after the maximal numbers of ^{125}I-ScTX binding sites have been reached in these brains, there does not seem to be a role for the myelin sheath in inducing the appearance of Na channels. In addition, evidence suggests that ^{3}H-STX uptake is not significantly different in brain and spinal chord from control or from amyelinated mutant rats (Oaklander, Pellegrino & Ritchie, 1984). Indeed, some workers have suggested that the nodal specialization, of which Na channel incorporation is a part, preceeds and may direct myelination (Waxman & Foster, 1980).

However, glial cell birth does represent an increase in non-neuronal tissue during the time course of this study (Berwald-Netter et al., 1982) which indicates that if the ^{125}I-ScTX or ^{3}H-STX binding was expressed on a per neuron basis, the adult levels of toxin binding would exceed the fetal levels by an even larger amount than is indicated by the per mg protein expression of our data. Refering to Table 7.1, this would mean that numbers of toxin binding sites per neuron are still increasing

between days 21 and 40 since the numbers per mg protein remain constant while the non-neuronal contribution to that protein increases.

None of the evidence reviewed to this point supports a clear role for neuronal activity, at least as measured by synapse formation, or neuronal-glial interactions in inducing the initial incorporation of Na channels although, due to the difficulty of assessing the numbers of Na channels per neuron, neither of these posibilities can be ruled out. Alternatively, the developmental expression of Na channels by the neurons may be intrinsically regulated or, as has been found for muscle, the initial incorporation of channels may be intrinsic but the expression of the full complement of channels may be dependent on input from a presynaptic cell (Sherman & Catterall, 1982).

The second aspect of the development of Na channels in rat brain is the maturation of the channels, measured as the appearance of high affinity ^{3}H-STX binding sites. In muscle, this transformation appears to depend on the presence of the presynaptic neuron, since muscles grown in aneural cultures don't develop TTX-inhibitable Na currents (Frelin et al., 1984). The maximal increase in numbers of ^{3}H-STX binding sites precedes the maximal increase in synapse density in rat brain (see above). However, as most neurons form many contacts, the time of maximal synaptogenesis may not be well correlated with the initial

onset of synaptic activity and therefore, neuron-neuron interactions may be important for regulating the maturation of the Na channel in neurons.

In these experiments, Na channel numbers, measured by the high affinity binding of the neurotoxins ^{3}H-STX and ^{125}I-ScTX, were low at birth and increased with age. The time course for the development of ^{3}H-STX binding sites was very similar in rat brain and muscle (cf Chapter 5), although the environments of the 2 cell types are very different. Maximum numbers of ^{125}I-ScTX binding sites were attained well before numbers of ^{3}H-STX sites. This was interpretted as evidence for the existence of an immature form of the Na channel binding ScTX but not STX. Although no definite conclusion can be made about factors which could be mediating the expression and maturation of the Na channel, neither myelination nor neuronal activity seem likely to be involved in initiating the expression of channels but it is possible that activity may be important for the appearance of the adult form of the Na channel.

CHAPTER EIGHT: COMPARISON OF THE EFFECTS OF BOTX IN ADULT AND NEONATAL RATS: NEUROMUSCULAR BLOCKADE AND TOXICITY.

8.1 Introduction

The ability of BoTX to block cholinergic transmission has been known for some time (Ambache, 1949) and its toxicology has been extensively studied (Ingram & Roberts, 1967). However, because much of the interest in BoTX toxicology was directed towards the problems arising from botulism as a clinical disease in humans and agricultural animals, most of this work studied the lethality of BoTX not its efficacy in blocking neuromuscular transmission. In addition, as much of the botulism arose from contaminated food, the most common routes of drug administration used in these studies were intra-peritoneal (ip) or intra-gastral (ig). Studies comparing the effects of BoTX in adult and neonatal animals were particularly likely to use the ig route because of the interest in infant botulism. This condition appears to arise due to colonization of the infant intestine by spores transmitted via the mother's milk (Moberg & Sugiyama, 1980).

BoTX has been widely used in studies of neuromuscular transmission (see for example: Mathers & Thesleff, 1978; Brown et al., 1982; Sellin & Thesleff, 1981; Bambrick & Gordon, 1986b). However, although the toxin is often administered subcutaneously (sc) in studies of neuromuscular blockade, less work has been done on the toxicology of BoTX by this route. In addition, most

studies have measured the acute (LD_{50}, time to death) rather than the chronic (muscle weight changes, duration of neuromuscular blockade) effects of the toxin. In one study where the chronic effects of BoTX-blockade were examined in neonatal rats and compared with the effects in adult rats (Brown et al., 1980), it was reported that, in rats treated with BoTX earlier than 21 days after birth, neuromuscular transmission was recovered more rapidly (within about 4 days) than in adult rats. The study concluded that this recovery was due to a return of polyneuronal innervation in the juvenile rats. Any dose dependence of the duration of the neuromuscular blockade was not discussed.

The purpose of this study was to determine the chronic effects of sc administered BoTX in neonatal as compared to adult rats with regard to: a) the duration of neuromuscular blockade; b) systemic effects of the toxin, such as weight loss and mortality.

8.2 RESULTS

8.2.1 Effectiveness of BoTX in blocking neuromuscular transmission. BoTX was delivered to the triceps surae (t.s.) muscles as a subcutaneous injection just above the knee in a minimum volume for all rats. Doses of BoTX ranging from 14-58 ng/kg (424-1758 mouse LD_{50}'s) rat weight were chosen to bracket the dosage of 12.5 mouse LD_{50}'s (equivalent to 625 mouse LD_{50}'s for a 20g neonatal rat) used by Brown et al. (1980).

The effectiveness of the resultant blockade was assessed qualitatively by monitoring the absence of two reflexes - the foot withdrawl reflex in response to toe pinch and the toe spread reflex (described in Section 2.2.3). St.-Pierre & Gardiner (1985) found good agreement between their results from similar observations and simultaneous recording of EMG activity. Prior to muscle dissection, blockade was also assessed in anaesthetized rats by testing whether suprathreshold stimulation of the sciatic nerve would elicit noticeable muscle contraction. In animals where neuromuscular transmission was blocked, both reflexes were abolished and indirect stimulation did not elicit any muscle activity. A muscle was considered to be partially blocked if there was any reflex response or muscle response to nerve stimulation but no attempt was made to quantitate the degree of partial block.

In comparison with its effects on adult rats, BoTX was dramatically ineffective in producing blockade of neuromuscular transmission in neonatal rats. In addition, no neonatal fatalities occurred even at doses of BoTX per kg high enough to kill two-thirds of the adult rats so treated by 2-3 days after injection. As death following BoTX injection is due to respiratory paralysis, the lack of neonatal mortality is additional evidence that neuromuscular transmission is more resistant to blockade by BoTX in juvenile than in mature rats. Fig. 8.1 summarizes the effectiveness of a range of doses of BoTX

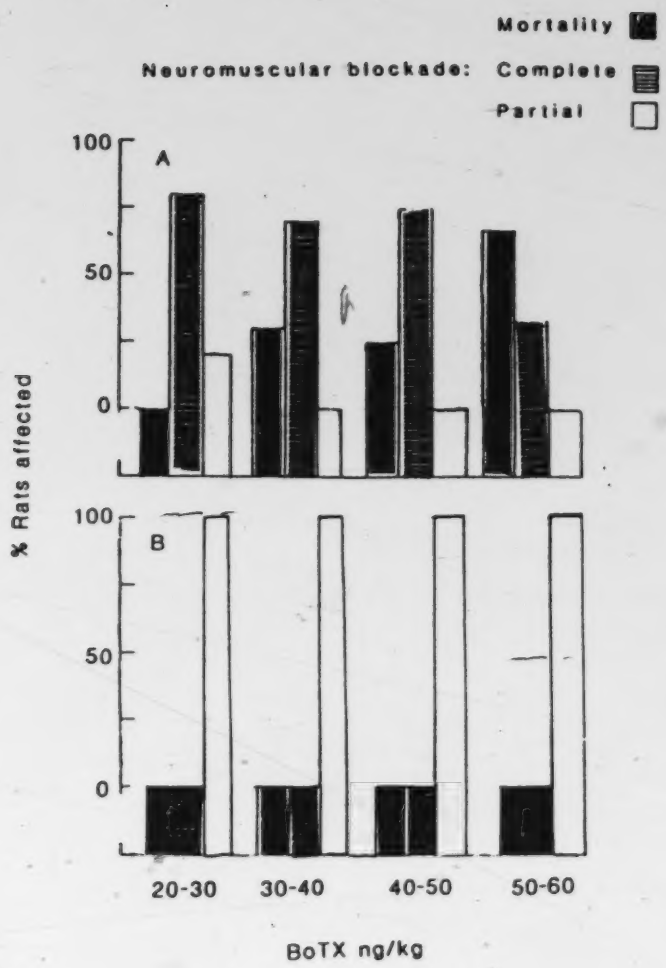


Fig. 8.1. BoTX dose-response relationship for mortality (stipled bars) and neuromuscular blockade: complete (striped bars) and incomplete (open bars) in A. Adult and B. 2 week old rats, one week after BoTX injection. The neonatal rats were not blocked 1 week after treatment at any dosage examined.

in producing neuromuscular blockade 1 week after injection for rats 2 or 8-10 weeks old (adults) at the time of treatment. For doses increasing from 20 to 60 ng/kg, which caused complete blockade for >80% of adult muscles and increasing morbidity among adult rats; all neonatal muscles were only partially blocked and no neonatal fatalities occurred. A similar ineffectiveness for BoTX in blocking neuromuscular transmission was observed for rats 1 and 3 weeks of age at the time of toxin injection.

8.2.2 Duration of BoTX blockade. At doses of BoTX above 20 ng/kg, neuromuscular transmission in the neonatal rats was blocked but the blockade only persisted for a short period of time. Even at the highest doses used, 60 ng/kg, neuromuscular blockade was only maintained for 3-4 days. The duration of blockade after a single injection of BoTX apparently increases with age since in adult rats, 8-12 weeks of age, complete blockade of neuromuscular transmission could be maintained for 11-14 days and partial blockade persisted for at least 20-21 days. In rats 1-4 weeks old, complete blockade was never maintained for longer than 4 days although incomplete blockade was observed more than 1 week after injection. Partial blockade might have persisted past this time but neonatal rats were not monitored for more than 9 days. It is important to note that, according to the same criteria as used for the adults, neuromuscular transmission in these neonatal rats was initially fully blocked. The difference

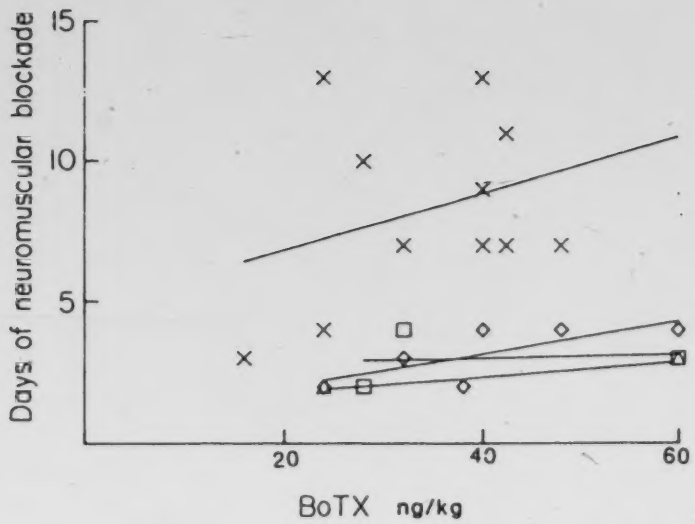


Fig. 8.2. Duration of neuromuscular blockade as a function of BoTX dose for rats 1 week (◇), 2 week (△), 3 week (□) and 7 or more weeks (×) of age at the time of treatment. There was no correlation between dosage and duration of neuromuscular blockade for neonatal rats (slopes of the linear regression lines were not significantly different from zero) and the duration of blockade in the adult rats (7 week of age and older) was only weakly dose dependent (RO=0.4).

between the juvenile and mature animals was not in the initial efficacy of the toxin but in the speed with which the younger animals recovered from its effects.

As is shown in Fig. 8.2, the duration of BoTX blockade in adult rats was very weakly dose dependent. (RO=0.4). The principle effect of increasing the BoTX dose was to increase the initial effectiveness of BoTX in blocking neuromuscular transmission, with increasing dose, the number of animals in which blockade was complete increased and the number of animals incompletely blocked correspondingly decreased.

8.2.3 Systemic effects of BoTX. Once neuromuscular transmission was completely blocked, increasing the dose of BoTX did not increase the local efficacy of the toxin as measured by the duration of neuromuscular transmission blockade, but did progressively result in increased mortality among adult animals and weight loss by adult and neonatal rats. The dose dependence of mortality was shown for adults in Fig. 8.1. BoTX in these doses did not result in any neonatal deaths. Loss of body weight was dose-dependent in both adult and juvenile rats. Table 8.1 compares rat weight loss for adult and 2 week old rats as a function of BoTX dose 1 week after injection. Adult rat weight decreased progresively with increasing dose to 78% of control at a BoTX dose of 50-60 ng/kg. Neonatal rat weight was less affected at lower doses but decreased to 60% of age matched control rat weight one week after

BoTX dose ng/kg	Rat weight % control	
	Adult	Neonate
0.0	100	100
25	90	100
30	88	75
40	83	70
50-60	78	60

Table 8.1. The effect of increasing doses of BoTX on adult and 2 week old neonatal rat weight one week after treatment as a percentage of age matched control rat weight.

treatment with 50-60 ng/kg BoTX.

8.3 DISCUSSION

The results of this study show that juvenile rats, aged 1-3 weeks are less susceptible to both the neuromuscular blocking and the lethal effects of BoTX than are adult animals.

The lesser efficacy of BoTX in neonatal, as compared to adult, rats is due to a decreased duration of effect, rather than due to a higher concentration of toxin being required to initiate neuromuscular blockade in neonates. This rules out the possibility that the lesser efficacy of BoTX in younger rats was due to their receiving lower absolute doses of the toxin. This could have been suggested as an explanation of these results since, in studies of the ip LD_{50} in mice, it was found that the absolute LD_{50} in mg/mouse did not vary with mouse weight. That is, the LD_{50} expressed as mg BoTX/kg body weight, appeared to decrease with increasing animal size (Lamanna, Jensen & Bross, 1955; Mills & Sugiyama, 1981). From this perspective the diminished efficacy of the BoTX in neonatal rats in this study would have been due to the administration of much lower absolute doses to the infants. On the other hand, Lamanna & Hart (1967) showed that rats differ from mice in that, in the former, the lethal dose of BoTX does vary with weight. The LD_{50} for small rats (125g) being 5/9 that for large rats (269g). Other differences in the response of mice and rats to BoTX

have been reported. Following BoTX injection, ultraterminal sprouts are observed in mouse but not rat soleus muscle (Pestronk & Drachman, 1985). In addition, some of the 3 week rats in this study received doses of 55 ng/kg, or 45 ng/rat. Adult 200g rats receiving 22 ng/kg, or a total dose of 44 ng BoTX, were blocked in 74% of the cases whereas in 7 trials none of the younger rats were completely blocked. Thus these results are in agreement with earlier reports of differences in the response of rats and mice to BoTX.

Several explanations may be advanced to explain the differential effect of BoTX on rats of different ages: a) BoTX may be taken away from the site of action and metabolized more quickly in the neonatal rats; b) BoTX may dissociate from its presynaptic binding site in neonates much more rapidly than in adults; c) the BoTX-binding site complex may turn over more swiftly in neonatal than in adult terminals; d) neonatal neuromuscular transmission may be more quickly restored due to more active nerve terminal sprouting by juvenile than by mature nerves or e) neuromuscular transmission may be restored by the return of nerve terminals which had been withdrawing as polyneuronal innervation was being eliminated in the developing rats. Brown et al. (1980) reported that the nerve terminals withdrawing from developing muscles during the first postnatal weeks (Redfern, 1970) return after BoTX treatment and that this is the principle means of

recovery of neuromuscular transmission from BoTX blockade in neonatal rats. Although it is not implausible that several factors, including, perhaps, an increased turnover of the BoTX binding protein on the presynaptic membrane, may contribute to the changes in BoTX toxicity with age, the re-establishing of polyneuronal innervation appears to be sufficient to explain the phenomenon.

A corrolary of the restoration of neuromuscular transmission after BoTX blockade by the return of polyneuronal innervation in neonatal rats is that the withdrawing nerve terminals must no longer express the presynaptic protein which binds BoTX. This is interesting as it demonstrates that the nerve-muscle contact is important in maintaining the specialization of the neuronal as well as the muscle membrane.

Increasing the BoTX dose beyond the dosage used by Brown et al. (1980) did not prolong the duration of neuromuscular blockade but did result in increased rat weight loss. In adult rats, complete neuromuscular blockade persisting for over a week was produced by the administration of a dose of BoTX of 40 ng/kg rat weight. This dosage resulted in a reduction of rat weight to 83% of control weight by one week. This would reduce a 220g rat to 183g. For the purposes of a developmental study, this is not an important reduction as a 183g rat is still a fully mature adult. However, in 2 week neonatal rats, a dosage of 60 ng/kg, which results in no fatalities and a

maximum duration of blockade of 4 days, reduced rat weight to 60% of age matched controls by 1 week. Therefore, when these rats are 3 weeks old and control rat weight is 53 g, BoTX treated rat weight is 32g. This is equivalent to the weight of a 16 day control rat. For developmental studies, the difference between a 16 and a 22 day rat can be considerable; therefore caution must be exercised in the interpretation of results for BoTX-blockade in neonatal rats at high toxin doses.

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